Transformation of *Nicotiana benthamiana* with different BWYV (*Beet western yellows virus*) sequences to test for virus resistance

Transformation von *Nicotiana benthamiana* mit verschiedenen Sequenzen des BWYV (*Beet western yellows virus*) zur Virus Resistenztestung

Von der Gemeinsamen Naturwissenschaftlichen Fakultät
der Technischen Universität Carolo-Wilhelmina
zu Braunschweig

zur Erlangung des Grades einer
Doktorin der Naturwissenschaften
(Dr. rer. nat.)

genehmigte
Dissertation

von
Sofia Valenzuela Aguila
aus Chile

1. Referent : Prof. Dr. R. Cerff
2. Referentin: Frau Prof. Dr. Renate Koenig
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Abbreviations

ACMV	*African cassava mosaic virus*
AlMV	*Alfalfa mosaic virus*
Amp	ampicillin
ArMV	*Arabis mosaic virus*
asRNA	antisense ribonucleic acid
BA	benzyladenine
BMV	*Brome mosaic virus*
BNYVV	*Beet necrotic yellow vein virus*
bp	base pairs
BWYV	*Beet western yellows virus*
BYMV	*Bean yellow mosaic virus*
°C	degrees Celsius
ca.	circa
CaMV	*Cauliflower mosaic virus*
CCMV	*Cowpea chlorotic mottle virus*
CFDV	*Coconut foliar decay virus*
CMMV	*Chrysythemum mild mottle virus*
CMV	*Cucumber mosaic virus*
CP	coat protein
CPMR	coat protein mediated resistance
CYVV	*Clover yellow vein virus*
CyRSV	*Cymbidium ringspot virus*
DEPC	diethylpyrocarbonat
DIG	digoxygenin
DMSO	dimethylsulfoxide
DNA	desoxyribonucleic acid
E. coli	*Escherichia coli*
EDTA	ethylendiaminotetraacetic acid
e.g.	for example
ELISA	enzyme linked immunosorbent assay
et al.	and others
GDD	Gly-Asp-Asp motif
GFP	green flourescent protein
GCMV	*Grapevine chrome mosaic virus*
GRSV	*Groundnut ringspot virus*
i.e.	that is
INSV	*Impatiens necrotic spot virus*
kb	kilobase
kDa	kilo Dalton
Km	kanamycin
min.	minutes
MCMV	*Maize chlorotic mottle virus*
MDMV	*Maize dwarf mosaic virus*
MOPS	4-morphoinpropansulfonic acid
MP	movement protein
mRNA	messenger RNA
NAA	naphthalene acetic acid
nm	nanometers

NPTII	neomycinphosphotransferase II
nt	nucleotides
OD	optical density
ORF	open reading frame
ORSV	*Odontoglossum ringspot virus*
PAMV	*Potato aucuba mosaic virus*
PBS	phosphatebuffered saline
PCR	polymerase chain reaction
PDR	pathogen derived resistance
PeaMV	*Pea mosaic virus*
PEBV	*Pea early browning virus*
PLRV	*Potato leafroll virus*
PMMV	*Pepper mild mosaic virus*
PPV	*Plum pox virus*
PRV	*Papaya ringspot virus*
PSbMV	*Pea seed-borne mosaic virus*
PTGS	post-transcriptional gene silencing
PVM	*Potato virus M*
PVS	*Potato virus S*
PVX	*Potato virus X*
PVY	*Potato virus Y*
Rep	replicase gene
Rif	rifampicin
RMR	replicase mediated resistance
RNA	ribonucleic acid
rpm	rounds per minute
RSV	*Rice strip virus*
r.t.	room temperature
RT-PCR	reverse transcriptase PCR
RTBV	*Rice tungro bacilliform virus*
SCMV	*Sugarcane mosaic virus*
SDS	sodiumdodecylsulphate
SLRSV	*Strawberry latent ringspot virus*
SMV	*Soyabean mosaic virus*
SSC	sodium chloride sodium citrate
TAV	*Tomato aspermy virus*
TBSV	*Tomato bushy stunt virus*
T-DNA	transferred DNA
TEV	*Tobacco etch virus*
TGMV	*Tomato golden mosaic virus*
TGS	transcriptionally gene silencing
TMGMV	*Tobacco mild green mosaic virus*
TMV	*Tobacco mosaic virus*
ToMV	*Tomato mosaic virus*
T-RNA	transgene RNA
TRV	*Tobacco rattle virus*
TSV	*Tobacco streak virus*
TSWV	*Tomato spotted wilt virus*
TVMV	*Tobacco vein mottling virus*
TYLCV	*Tomato yellow leaf curl virus*
TYMV	*Turnip yellow mosaic virus*

U	Unit
VPg	genome linked viral protein
vRNA	viral RNA
WClMV	*White clover mosaic virus*
WMVII	*Watermelon mosaic virus II*
wpi	weeks post infection
ZYMV	*Zucchini yellow mosaic virus*

1 INTRODUCTION

It is estimated that about 10% of the world crop production is lost annually due to viral, bacterial and fungal diseases. Although most plants are resistant to or can hardly be infected by viruses, plant viruses gained a significant impact in agriculture being nowadays the second cause of plant diseases (Matthews, 1992).

Viral diseases of crop plants constitute a major economic problem through reduction in product yield (Zaitlin and Hull, 1987). However it can not be denied that most of the serious diseases affecting plants have been a direct or indirect result of human activity, among them: introduction of virus infected seeds or vegetative material in new areas, introduction of virus vectors in new areas, and introduction of a new crop in an area when that crop is susceptible to a virus already present in this region (Matthews, 1991). Other factors can be mentioned such as monocultures, which make crops highly attractive for specific pathogens. In the last years breeding programs have been mainly focused on gaining higher yields and improved product quality, rather than resistance to pathogens (de Haan, 1998).

Mainly three strategies have been used to avoid viral diseases, i) removal of virus reservoirs, i.e. infected plants, ii) preventing viral spread from plant to plant by eliminating vectors and iii) to produce and grow virus-resistant cultivars (Matthews, 1992). The use of virus-free starting material and eradication of infected plants means that one must be able to detect viruses at an early stage. Most of these methods are expensive and the chemicals to control the presence of viruses can be damaging to the environment and consumers health (van den Boogaart et al., 1998). Therefore the production of virus-resistant cultivars has increased in the last years especially due to public pressure to avoid the use of pesticides.

The term resistance in plant breeding has been used not only or necessarily to imply absolute immunity, but also includes a delay in the symptoms and/or a milder form of infection (van der Boogaart, 1998). In the past confusion arose among the terms resistance and tolerance. The terminology proposed by Cooper and Jones (1983) deals with virus-host interactions in the individual plant. In their proposal a plant is either infectible or immune (not infectible). An infectible plant is classified as susceptible if specific viral functions required for virus survival in the plant proceed with relatively little or no restrictions. It is classified as resistant if these functions proceed with considerable restriction. The plant is classified as tolerant if its symptoms response is mild, or as sensitive if symptom response is severe. Symptom response may, but does not necessarily, reflect the degree to which viral functions are restricted (Cooper and Jones, 1983). Therefore, resistance of plants to infection can be defined as the ability of the plant to reduce substantially the amount of virus replication and spread. However, this term must always be used in a “temporal” way,

since during history it has been shown that it is always possible that a mutant of a virus can arise and overcome the plant resistance (Matthews, 1992).

Zaitlin and Hull (1987) have proposed that resistance can work at three levels, i) extreme resistance, known as immunity, where no viral replication takes place; ii) in some cases virus may replicate, but can not spread from cell to cell and iii) in some hosts viral replication and spread is allowed, but can induce a hypersensitive response of the plant, restricting the virus to a region around the point of entry.
When no genetic source for resistance can be found the use of tolerant crops can be helpful, however it is not the best solution, since it can act as a reservoir of viruses, or a double infection with an unrelated virus can occur, leading to severe disease (Matthews, 1992).

Cross protection has been one of the most employed methods to obtain virus-resistant crops. The term cross protection is now widely accepted for cases in which the protecting virus spreads systematically in the host (Fraser, 1998). Cross protection is a phenomenon in which a plant that has been infected with a mild strain of a virus that produces no or few symptoms, is protected from superinfection by a severe strain of a related virus. It has been used in agriculture to protect crops for which no other source of resistance or control measures were available (Fulton, 1986). However, this technique has several disadvantages, firstly, the protecting mild strain might mutate to a more severe form, secondly, it may act synergistically with other viruses, thirdly, it may spread to other crops on which its effects may be more drastic. Finally, even though the mild strain does not induce severe symptoms on a crop, it may nevertheless reduce product yield of 5 to 10% (Buck, 1991; Fraser 1998). In addition some virus resistance genes are recessive and/or inherited in a complex manner (van der Boogaart et al., 1998).

Powell-Abel et al. (1986) suggested that most or all of these objections could be overcome if cross-protection was engendered in plants as the result of expression of a single viral gene, rather than as a result of infection with an intact virus. This work initiated a new research line, which enabled the use of recombinant DNA to produce genetically modified plants that were resistant to virus infection.

Since the middle of the 1980’s genetic transformation strategies have been applied to develop virus resistant plants through what has been referred to as “pathogen derived resistance“ (PDR). It is based on the use of transgenes derived from the genome of the targeted pathogen to develop resistance. The expression of genetic material in the plant will disrupt the essential pathogenic processes and hence result in resistance to the pathogen (Sanford and Johnston, 1985). Due to the easiness and accessibility to viral genomes compared to those of bacteria or fungal pathogens, this

Virus group	Virus	Chimeric gene	Transgenic plant	Challenged virus
Alfamo	AlMV	35S-CP-nos	Tobacco	AlMV
			Tomato	AlMV
Carla	PVS	35S-CP-nos	Potato	PVS, PVM
Cucumo	CMV	35S-CP-nos	Tobacco	CMV, CMMV
Furo	BNYVV	35S-CP-nos	Sugarbeet	BNYVV
Ilar	TSV	35S-CP-nos	Tobacco	TSV
Luteo	PLRV	35S-CP-nos	Potato	PLRV
Nepo	ArMV	35S-CP-nos	Tobacco	ArMV
Potex	PVX	35S-CP-nos	Potato	PVX
		35S-CP-rbcS	Tobacco	PVX
			Potato	PVX
Poty	PPV	35S-CP-35S	Tobacco	PPV
	PVY	35S-CP-rbcS	Potato	PVY
		35S-CP-nos	Tobacco	PVY
	WMV II	35S-CP-35S	Tobacco	WMVII, PVY
				TEV, BYMV
				PeaMV, CYVV
				PeMV
Tobamo	TMV	35S-CP-nos	Tobacco	TMV
			Tobacco	ORSV, PMMV
				TMGMV

Table 1. Some examples of CPMP. In most cases tobacco has been employed as a model plant. (Modified from Miller and Hemenway, 1998).

approach was first demonstrated with viruses. Recently, several strategies have been developed to engineer plants resistant to pathogens (Lomonossoff, 1995; Baulcombe, 1996a).

In 1986 Powell-Abel et al. demonstrated that transgenic tobacco expressing tobacco mosaic virus (TMV) coat protein (CP) showed resistance to TMV, which was seen as delay in symptoms in the transgenic plants. This phenomenon is nowadays referred to as coat-protein mediated resistance (CPMR), and usually confers resistance or immunity to plants. Since then many studies in which plants transformed either with full length or truncated CP derived from different plant viruses have been done (i.e. Silva-Rosales et al., 1994; Spillane et al., 1997; Barker et al., 1998; Sinisterra et al., 1999; McDonald et al., 1996; Hassairi et al., 1998). A summary of some of the CPMR that have been obtained up to date is shown in Table 1. This field has rapidly progressed from testing resistance in model plant systems under growth chamber conditions to conducting field trials on agronomically significant crops such as tomato, potato and sugarbeet among others (Miller and Hemenway, 1998).

In most cases the presence of the CP confers resistance to infection by closely related viruses but not to more distantly related or unrelated viruses. The protection provided by CP can be overcome by high concentrations of virus and it is less effective when virus RNA is used as infectious agent (Sturtevant and Beachy, 1993;

Fitchen and Beachy, 1993). The mechanism of CPMP in CP TMV tobacco plants has been recently reviewed by Beachy (1999).

Different strategies have been developed to improve the protection obtained by CP of several viruses. Barker et al. (1994) determined that combining PLRV CP and host resistance genes in potato gave additive effects on protection against PLRV. Some reports have shown that transgenic plants expressing potyviral CP genes were protected against heterologous potyviruses (Namba et al., 1992; Murray et al., 1993). An alternative approach to achieve broader resistance is transformation of multiple CP genes within one plant expression vector (Lawson et al., 1990). Cuozzo et al. (1988) transformed tobacco plants with the CP of CMV using the sense or antisense construct. They showed that the CP^+ plants were resistant to the virus, while those containing the antisense transgene were protected only at low levels of virus inoculation. Lindbo and Dougherty (1992) have demonstrated that different mutated versions of the CP of TEV were more effective in conferring resistance than the full length gene. They postulate that this truncated form of TEV CP could interfere with the process of long distance movement.

When the CP gene is used as a target gene to confer virus resistance in plants, it is possible to detect low or high amounts of this protein in the plant cell, in most but not in all cases. It is postulated that the resistance obtained is due to a “dominant negative mutant“ (Hersowitz, 1987; Carr et al., 1992), i.e. the transgenic protein can interfere with the viral particle by breaking the equilibrium of some elements in the plant cell, since it can for example sequester cell components necessary for viral replication (Braun and Hemenway, 1992). When using a truncated form of CP or in a few cases Rep protein (Lindbo and Dougherty, 1992; Carr et al., 1994), and where the corresponding truncated protein has been detected, it has been postulated that this form can capture i.e. viral RNA, therefore inhibit the normal viral replication process.

Several reports using potyviruses have indicated that the CP levels were low in plants expressing CP sequences and frequently the lowest expressors were best protected (Kaniewski et al., 1990; Sudarsaono et al., 1995). It remains to be determined if the lower expression of proteins in some systems reflect a technical difficulty with expression of certain genes, stability of the protein products, or absence of viral factors required for expression and/or stability (Miller and Hemenway, 1998).

Some years after development of CPMP, transgenic plants were also engineered to express other viral sequences and genes, including asRNA, satellite RNAs, sense transcripts, defective-interfering (DI) sequences, protease genes, movement protein genes and replicase genes (some examples are shown in Table 2). As with CPMR, transgenic plants expressing these viral sequences display resistance phenotypes

ranging from delay in symptom development to apparent immunity. As an example, the MP from different viruses has been used either as full length or truncated form to transform plants and test for resistance (Ciuffreda et al., 1998; Beck et al., 1994; Cooper et al., 1995). In most cases it has been observed that MP-mediated resistant (MPMR) plants have been obtained using truncated dysfunctional MPs, suggesting that the modified gene operates in transgenic resistant plants as a dominant negative mutant (Ciuffreda et al., 1998; Herskowitz, 1987).

van Dun et al. (1988) transformed tobacco plants with a nonstructural viral gene, the viral polymerase of AlMV, which is encoded by RNA1 and RNA2 of the viral genome. They were able to detect the transgene as DNA and RNA, but they did not find the corresponding protein. In this case, plants were susceptible to virus infection, showing no difference in response compared with either non transgenic plants or vector transformed plants. The authors assumed that the lack of protection could be due to a low expression level of the integrated genes.

Golemboski et al. (1990) showed for the first time that it was possible to obtain resistant plants using a truncated form of the viral polymerase (54 kD) of TMV. Although their work was intended to study the function of this protein, they found that transformed tobacco plants were highly resistant to virus and RNA inoculation. This type of resistance is known as replicase mediated resistance (RMR), which is based on transgene expression of either the full-length or a truncated form of viral polymerases and it has been demonstrated in many studies with tobra-, cucumo- and potexviruses, among others (Palukaitis and Zaitlin, 1997; Baulcombe, 1996b).

The first report showing resistance in plants containing a copy of a full length polymerase was demonstrated by Braun and Hemenway (1992), who used the replicase gene of PVX, since then a few reports (Audy et al., 1994; Rubino et al., 1993; Sijen et al., 1995; Russo et al., 1998; Huet et al., 1999) have appeared. As with MPMR in most cases a truncated or mutated form of the viral replicase, seems to be more effective in conferring resistance against viral infection (Carr and Zaitlin, 1991; Taschner et al., 1991; Longstaff et al., 1993; MacFarlane and Davies, 1992; Guo and Garcia, 1997, just to name some).

In general rep-transformed plants operate against very high doses of inocula, either virus or viral RNA; resistance is not related to the transgene RNA levels and it is highly virus strain-specific (Tenllado et al., 1995). In some cases, the response has been shown to be dose-dependent (Anderson et al., 1992). Some plants transformed

Type of sequence/gene	Virus	Type of sequence/gene	Virus
Antisense		Replicase	AlMV
Coat Protein	CMV		CMV
	PLRV		CyRSV
	PVX		PEBV
	PVY		PVX
5' 3' ends of RNA	CMV		PVY
3' end of genome	TMV		TMV
5' end of genome	TMV	Movement proteins	TMV
Satellite	CMV		WClMV
	TRV	Protease	PVY
DI sequences	ACMV		TVMV
	BMV		
Sense transcripts			
Untranslatable CP gene	PVY		
	TEV		
	TSMV		
3' end of genome	TYMV		

Table 2: Examples of other mediated protected strategies which have been employed to assay for virus resistance in plants. (Modified from Miller and Hemenway, 1998).

with the replicase gene were not only fully susceptible to virus infection, but could also complement mutant viruses with defect genes (van Dun et al., 1988; Baulcombe, 1994; Lomonossoff, 1995). Canto and Palukaitis (1998) obtained tobacco plants that expressed an active 1a protein of CMV, which was able to complement the replication of RNAs 2 and 3, in absence of viral RNA1. The transgene did not only complement the replication of the heterologous viral RNA but also allowed long distance movement in the plant.

Transgenic plants containing the truncated 54Kd protein of TMV Rep have been widely studied. It has been indirectly demonstrated, by using protoplasts of resistant lines, that the expression of the protein is required to confer resistance (Carr et al., 1992; Lomonossoff, 1993). Similar evidence has been found by Brederode et al. (1995), with a mutated form of the AlMV RNA replicase gene.

Not all examples of resistance observed in RMR or CPMR can be explained by the interference of the transgene protein, in most works no transgene protein has been detected despite the use of the strong 35S promoter derived from CaMV (Longstaff et al., 1993; Baulcombe, 1996b). However, it has been postulated that in Rep transgenic plants, the protein can be either synthesized at a very low level or it can have a high turnover in the plant cells (Golemboski et al., 1990; Tenllado et al., 1995).

In a large number of studies an inverse correlation between the degree of resistance or immunity obtained in transgenic plants to a pathogen and the steady state level of the transgene mRNA has been observed (Prins and Goldbach, 1996; Mueller et al.,

1995; Lindbo et al., 1993). Therefore it is suggested that resistance does not require the synthesis of any virus-derived protein or protein fragment (Lindbo et al., 1993; van der Vlugt et al., 1992). In these cases the term RNA-mediated resistance or defense (RMD) is used (Dougherty at al., 1994). The resistance provided by the expressed RNA is usually strong, not being overcome by high doses of virus inoculum concentrations and it is highly virus specific (reviewed in van der Bootgaart et al., 1998; Bruening, 1998; Wassenegger and Pélissier, 1998). In many studies it has been observed that the most resistant plants show low steady state levels of transgene RNA (reviewed in Baulcombe, 1996b; Matzke and Matzke, 1995; Mueller et al., 1995) or high levels of transgenic transcript are detected (Brederode et al., 1995). Hellwald and Palukaitis (1995) have proposed that at least two different mechanisms could account for replicase mediated resistance, as studied with CMV. In the first case, the viral RNA would serve as the target for the resistance against CMV. In the second mechanism postulated by these authors, the replicase mediated resistance works against the viral movement. Similar results were observed by Nguyen et al. (1996).

Gene silencing (GS) in plants is manifested as decreased accumulation of specific mRNA and occurs most often when there are multiple copies of a particular sequence present in the genome. In transgenic plants the phenotype of a silenced transgene is maintained through vegetative propagation or organ regeneration and can be transmitted by grafting (Bruening, 1998). However the transmission to the progeny through meiosis is unpredictable, while silencing can appear with frequencies of 2-100%, and even more progeny of a non silenced plant may be silenced (reviewed by Bruening, 1998).

There are two major mechanisms of gene silencing i) those in which mRNA level is regulated transcriptionally (TGS) and ii) those in which it is regulated post-transcriptionally, also known as co-suppression (PTGS: reviewed by Stam et al, 1997; Depicker and van Montagu, 1997; Meyer and Saedler, 1996). In the case involving viral transgenes the mechanism is post-transcriptional and can be targeted in a sequence specific manner, against the transgene mRNA as well as the RNA genome of the virus (Smith et al, 1994; Guo and Garcia, 1997).

In TGS it is usually found that the promoter region is inactivated, in most cases associated with methylation, while in PTGS the promoter is active, but the mRNA does not accumulate and in some cases this has been associated with methylation of the transgene, especially in the 3’ end (Stam et al., 1997; English et al., 1996; Matzke and Matzke, 1998). However, recently, Jones et al. (1999) have demonstrated that PTGS and methylation can be uncoupled processes.

There are two main models to explain PTGS in transgenic plants:

a) Threshold model, where the plant can sense the transcriptional level of the transgene and if it is too high, it will proceed to degrade it (Smith et al., 1994). This would activate a host RNA dependent RNA polymerase, which would synthesize small fragments of asRNA or cRNA (complementary), therefore generating a dsRNA (double stranded) (Prins and Goldbach, 1996; Baulcombe, 1996b; Waterhouse et al., 1998).

b) Aberrant RNA (aRNA), in this case both the aRNA and normal homologous RNA will be degraded. For the synthesis of aRNA it involves a silencer locus and a receptor locus. The transcription of the silencer locus is not essential. Probably there is DNA-DNA pairing between the two loci, so the transcription of the receptor locus would be changed, leading to production of aRNA, that would induce a degradation mechanism for all homologous RNAs. This aRNA would act as a target for the RNA dependent RNA polymerase, giving rise to a double stranded RNA (Mueller et al., 1995; English et al., 1996; Waterhouse et al., 1998).

In both cases it is postulated that the dsRNA is degraded by RNases present in the cell (reviewed by van der Boogaart et al., 1998).

Although the exact mechanism by which PTGS operates is not known, various findings that viruses can both initiate and be targets of PTGS suggest that PTGS is a natural mechanism found in plants to recognise and combat foreign nucleic acids (Voinnet et al., 1999; Matzke and Matzke, 1998). Recently it was shown that PTGS involves systemic spread of a silencing signal directing a sequence specific RNA degradation (Kasschau and Carrington, 1998). To account for the sequence specificity and nature of PTGS, it has been proposed that asRNA forms a duplex with the target RNA, thereby promoting its degradation or interfering with its translation (Lindbo et al., 1993; Stam et al., 1997). Hamilton and Baulcombe (1999) have detected an asRNA complementary to the targeted mRNA in four different types of PTGS in plants, which had a uniform length of 25 nt. These are not degradation products of the RNA, since they have antisense polarity, but it could be processed from a larger molecule synthesized in the cell. Bucherna et al. (1999) have found that the presence of two copies per gene is essential for silencing, but that they can be present either at the same locus or different loci. By grafting it has been demonstrated that some silencing factors are involved in this process, which can move from one part to the other of the plant apparently between silenced and non-silenced plants, but not between silenced plants (Palauqui et al., 1997; Sonoda and Nishiguchi, 2000). If aRNA or cRNA can act as diffusible factors of silencing and whether they can move alone or complexed with ribonucleoproteins remains unknown (Vaucheret et al., 1998).

“Recovery” is the phenomenon whereby plants that have undergone an initial round of viral infection, nevertheless develop new healthy virus free leaves, being resistant to subsequent infection with the same or related viruses (Lindbo et al., 1993; Tenllado et al., 1995; Guo and Garcia, 1997). Ingelbrecht et al. (1999) have reported that sugarcane plants transformed with the CP of SCMV required variable time for recovery, ranging from months up to a year. In this case it is postulated that the virus triggers transgene silencing and virus resistance. The same has been postulated by Jones et al. (1998) who observed a recovery phenomenon in transgenic peas expressing the viral replicase (Nlb) gene of PSbMV. It is believed, that both transgene transcription and virus replication contribute to reach the level of accumulation of RNA that triggers PTGS (Stam et al., 1997; Selker, 1999), as postulated by the threshold model.

In at least some cases of RMR, however PTGS seems not to be the main cause of the resistance response. In the case of plants transformed with the replicase gene of AlMV (Brederode et al., 1995) the resistance obtained appeared to be protein mediated, and high steady-state levels of the transgene were found in resistant plants. In the case of employing CMV replicase gene (Carr et al., 1994; Canto and Palukaitis, 1998), virus replication was severely reduced but not completely suppressed as in PTGS, only limited cell to cell movement occurred and long distance movement was blocked. Canto and Palukaitis (1999) have reported that RMR in CMV does not directly block the trafficking of CMV RNA.

There have been a number of models proposed to explain PTGS involved with anitisense, co-suppression and viral resistance, but none explains all cases observed (van der Boogaart et al., 1998). In all models it is proposed that gene silencing and virus immunity involve a rapid degradation of RNA, that has a high degree of homology with the silencing transgene (Waterhouse et al., 1998). In some cases it has been suggested that a plant RNA-dependent RNA polymerase makes complementary strands from the transgene mRNA and these potentiate the degradation of the target RNA (Ratcliff et al., 1999). Lindbo et al. (1993) have proposed that this is triggered by high levels of transcription and that this correlated with high gene copy number. All these phenomena are still not well understood, and it seems to be even more complicated as thought in the beginning.

It has been recently reported that gene silencing can be induced by plant virus infections in absence of any known homology of the viral genome to host genes and that this silencing may occur at the transcriptional or posttranscriptional level (Ratcliff et al., 1997; Covey et al., 1997). These authors have shown that non transgenic kohlrabi (*Brassica oleracea gongylodes*) and oilseed rape (*Brassica napus*) plants show initially systemic symptoms when infected by CaMV, from which they recover completely by loss of the virus. This “recovery phenomenon” correlates with the lack of accumulation of CaMV 19S and 35S RNA, although rates of transcription remain

unchanged (Al-Kaff et al., 1998; Covey et al., 1997). Therefore it seems possible that plants can naturally escape virus infection in a post-transcriptional manner. On the other hand, a viral sequence that is able to suppress gene silencing in plants has been recently found (Anandalakkshmi et al., 1998; Ruiz et al., 1998; Kasschau and Carrington, 1998; Voinnet et al., 1999).

The RNA mediated resistance mechanism has advantages in the sense that it is not overcome by high virus inoculations and there is no viral protein synthesis, therefore avoiding any risk of encapsidation. But as well, the high sequence specificity arises the question if the resistance will be durable (van der Boogaart et al., 1998). Hellwald & Glenewinkel (1999) have shown that RMR against CMV, which is highly effective against the donor virus Fny-CMV and other subgroup I strains of CMV, but not against subgroup II strains (Anderson et al., 1992; Zaitlin et al., 1994), can be overcome by strains which have ca. 92% homology with the donor strain.

The use of virus resistant transgenic crops has several advantages, i.e. it is possible to introduce virus resistance in susceptible varieties without affecting the intrinsic properties of that cultivar and it provides an alternative source of virus resistance, which is of particular utility when a host resistance is either unavailable or of difficult access. From the environmental point of view it would greatly reduce the use of pesticides, normally applied for the control of virus vectors.

Despite these advantages, many authors have pointed that care must be taken before releasing these plants into the field (reviewed by Aaziz and Tepfer, 1999; Rubio et al., 1999; Hull, 1998). There is some discussion among potential risks associated with virus-derived resistance, transgene escape, gene flow between crops and wild plants or soil bacteria. Recombination and complementation are of most concern.

Recombination, which is the physical joining of sequences from different sources, viral or non viral, can result in large-scale change to RNA virus genomes, leading to viral evolution. Two different mechanisms seem to be involved, reassortment, in multipartite viruses and recombination can occur in either segmented or unsegmented viruses, when “donor“ nucleotide sequence is introduced into a single, contiguous acceptor RNA molecule (reviewed by Worobey and Holmes, 1999; Aaziz and Tepfer, 1999). Recombination has been demonstrated for a large number of plant viruses, among them luteoviruses (Gibbs and Cooper, 1995). In isolates of PLRV the 5´ terminal region of a Scottish isolate was homologous to the tobacco chloroplast ORF 196 (MacFarlane and ref. therein, 1997).

The possibility of recombination between viral RNA and transgenic plants has been demonstrated in a few cases under high selection pressure using CCMV (Greene and Allison, 1994). Some examples have shown as well that the pathogenicity of the

virus can be affected after recombination events when using transgenic plants and a defective mutant (i.e. CaMV with CMV/TAV isolate, Schoelz and Wintermantel, 1996; Király et al. 1998). Recently, Borja et al. (1999) have demonstrated restoration of TBSV, using CP transgenic plants, with a mutant virus. However, they postulate that in all cases where recombination in transgenic plants has been observed, plants are not resistant to infection, and the experimental conditions were optimal for detection of recombinants. According to these results it can be shown that recombination can occur between transgenes and defective viruses. However, the conditions of high or moderate pressure used, do not represent what would be expected to occur under field conditions (Rubio et al., 1999). It must as well be considered that virus replication is greatly reduced in highly resistant transgenic plants, therefore the recombination frequencies should as well be less (Baulcombe, 1996a; Wilson, 1993). The use of transgenes that contain relatively small segments of non translatable viral genes fused to non homologous sequences, could minimise the frequency of viral recombination (Rubio et al., 1999). The exchange of a replicase gene or part of a replicase gene from one virus to generate another virus in the same genus has usually led to either a non-functional or a poorly adapted virus (Palukaitis and Zaitlin, 1997).

Complementation, the process by which a functional gene of one virus corrects for defectiveness in the same function of another coinfecting virus, is a well known phenomenon. Thus, expression of a transgene could induce susceptibility in the transgenic plant to new viruses, if the expressed gene provided an essential function that the new virus could not provide by itself (Kaniewski and Thomas, 1998).

Most of the resistance tests of transgenic plants are performed under greenhouse conditions, there are few examples of field studies (Kawchuk et al., 1997). Obviously, performing the assays in a controlled glasshouse has advantages from the scientific point of view, however it has been demonstrated that when plants are tested in the field, different responses are observed as those obtained under controlled conditions (Kanieswski and Thomas, 1998). Some authors have postulated that the environment plays an important role in the resistance observed with some transgenic plants (Barker et al., 1998). In some cases it has been demonstrated that resistance is temperature dependent (Neijidat and Beachy, 1990), therefore studies must be extended to fields, where a greater variety of soil and climatic conditions as well as sites with high incidences of natural infection can be found (Kanieswski and Thomas, 1998).

Luteoviruses are an important family of viruses, which can infect a wide range of hosts, causing important losses in agriculture. The name luteovirus is derived from the Latin root for yellow, reflecting the tendency of members of the group to induce yellowing symptoms. The family *Luteoviridae* was recently proposed (D'Arcy and Mayo, 1997), allowing to classify each of the two former subgroups of the genus

Luteovirus in two new genera *Luteovirus* (MAV) and *Polerovirus* (BWYV or PLRV) (Martelli, 1997; Pringle, 1999; Fauquet and Mayo, 1999).

Beet western yellows polerovirus (BWYV) was firstly reported in California (Duffus, 1960). In Europe it was first reported by Gilligan et al. (1980) in oilseed rape (*Brassica napus*). Stevens et al. (1995) state that differences should be made between BMYV and non-beet-infecting isolates of BWYV. They analysed aphids caught in either sugar beet or oilseed rape fields in England and studied for the presence of BMYV and/or BWYV. Although in a few cases they detected sugarbeet plants infected with BWYV, they were quite few compared to the high number of aphids carrying the virus, therefore they assume that not all isolates of BWYV are able to infect sugarbeet. Graichen and Rabenstein (1996) made a study of host range of BWYV and BMYV isolates, where they found that none of the BWYV isolates was able to infect sugar beet plants, but it could infect *Brassica* species, therefore the authors proposed to rename the virus as *Turnip yellows virus* (TuYV). In this work the name of BWYV will be used as synonym of TuYV. The virus is probably distributed world wide, it spreads systemically, has a wide host range, infecting more than 150 species in 23 dicotyledonous families, including economically important crops such as *Spinacia oleracea*, *Laetuca sativa* and *Brassica napus*.

Field infections of crops usually originate from weed species like *Senecio vulgaris* and *Capsella bursa-pastoris*, which act as natural overwintering hosts of the virus. The virus is phloem specific, occurs at low levels in its hosts and is obligatory transmitted by aphids in a persistent circulative manner. *Myzus persicae* (Sulz.) is probably the most efficient and important vector under natural conditions (Casper, 1988). The minimal time for viral acquisition has been reported to be 5 minutes, this is followed by a latent period of at least 12 hours and the virus can be transmitted with an inoculation access period of 10 to 30 minutes (Casper, 1988). However these times are dependent on many factors, such as efficiency of the vector, virus concentration in the plant host, virus strain, temperature and other environmental factors. Typical symptoms due to infection are induction of chlorotic symptoms, which are typically first observed at the tips or margins of leaves and soon over the entire leaf. Intervenial chlorosis occurs in older leaves and usually it ends with necrosis. However, rapid collapse of infected tissues, necrosis and severe stunting are not uncommon among BWYV hosts. With the impairment or loss of chlorophyll, anthocyanin accumulates in the leaves of some BWYV-infected plants resulting in reddish patterns (Hampton et al., 1998).

In Germany regional differences in the infestation degree from TuYV in oilseed rape have been detected (Graichen et al., 1997). The authors found that in the northern, western and eastern part of Germany in average 71% of the plants tested were infected by TuYV, while in the southern part of Germany this value was ca. 15%, in different fields during the period 1995/96.

The genome of luteoviruses comprises a single stranded RNA molecule of positive polarity. The 5' end the viral RNA is covalently bound to a genome linked protein (VPg). The complete genome of BWYV has been determined (Veidt et al., 1988; Reutnauer et al., 1993; Ziegler-Graff et al., 1996). It consists of ca. 5,600 nucleotides, which are arranged in 6 open reading frames (ORF0 to ORF5, see Fig 1).

The genes in the 3' half of the genome are expressed from a subgenomic RNA (sgRNA), having at the 3' end a high homology to each other, probably because they confer the properties of circulative aphid transmission and phloem specific cell to cell movement (Miller et al., 1997). Recently it has been postulated that a new ORF (ORF7) at the 3' end could be present in BWYV, as a product from a sgRNA2, which has been found in PLRV (Ashoub et al.;1998).

ORF0 seems to be involved in viral symptom expression, as determined for PLRV (van der Wilk et al.; 1997). The ORF1 and ORF2 encode the viral replicase complex, having ORF1 the helicase activity, while ORF2 contains the replicase motif, characterized by the presence of the highly conserved GDD motif. Members of *Luteovirus* (former subgroup I) show a high percentage of identity in the sequence motifs of their putative RNA polymerase with carmoviruses and tomobusviruses, while *Polerovirus* (former subgroup II) show a high identity percentage with sobemoviruses (Habili and Symons, 1989).

The major capsid protein of BWYV is encoded by ORF3 and corresponds to a 22.5 kDa. The minor capsid protein is a readthrough protein P74 derived from ORF3 (Wang et al., 1995) and the adjacent ORF5 by translational readthrough of the ORF3 codon (Veidt et al., 1988). This protein is required for efficient virus accumulation, since it intervenes in virus movement, increasing the rate of new infection foci (Brault et al., 1995; Mutterer et al., 1999). The product encoded by ORF4 (19 kDa) has been suggested to be a MP-like protein, comparing with results observed in PLRV (Tacke et al., 1991; Schmitz et al., 1997).

Since the virus is obligatorily transmitted by aphids, the major strategies for its control are:
i) elimination of virus source plants, ii) application of insecticides, iii) adjustment of the time for planting to avoid maximum aphid populations and iv) planting cultivars resistant or tolerant to field infection (Matthews, 1991).

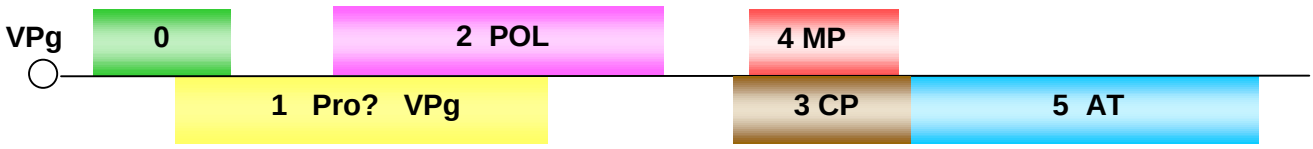


Figure 1 Genome organization of *Polerovirus*. POL: RNA-dependent RNA polymerase; PRO?: is the putative protease; VPg: genome-linked protein; CP: coat protein; MP?: putative movement protein; AT: read-through domain of the coat protein gene probably required for aphid transmission.

Aphids are the most important group of plant virus vectors in temperate regions, transmitting a large number of different viruses. The life cycle of many aphids is rather unusual and quite complex. Overwintering is done in the egg stage, and eggs hatch in the spring into females that reproduce parthenogenetically and give birth to living young. The first and second generation usually consist of wingless individuals, but eventually winged forms migrate to a different host plant. Large populations of aphids can be built in a short time, but some nature parasites, ladybird beetles, lacewings and larvae of certain syrphid flies, prevent their dissemination (Barror et al., 1976). The application of insecticides is useful to kill wingless aphids before viral transmission occurs, however if winged aphids arrive carrying virus, insecticides are of little value in preventing transmission (DiFonzo et al., 1995 and references therein).

When an aphid starts feeding on a leaf, it first produces a drop of gelling saliva where after its stylets penetrate in the epidermis. Subsequently the aphid proceeds to deeper cell layers until it finally reaches the phloem sieve tubes from which it derives its nutriment. As most of the circulative and persistent transmitted propagative viruses are restricted to phloem tissue, these viruses are usually acquired only in longer feeding periods (Dijkstra and de Jager, 1998).

It is interesting to note that up to date no real resistance (as defined above) against luteoviruses has been found. It has been postulated that the inhibiting RNA or CP are not present in cells where these viruses replicate (de Haan, 1998). Although many attempts have been made using the CP of PLRV, the results of these studies only show a lower average value of the ELISA from some transgenic plants compared to those observed in the infected controls. Gielen et al. (1994) transformed lettuce plants with the CP of BWYV, but unfortunately no resistance was observed in any of the lines tested.

It can be postulated that due to the phloem specificity of this virus, it should be difficult to obtain transgenic plants with resistance against BWYV, using normal plant promoters. For phloem-specific viruses expression of virus resistance genes is desirable since constitutive expression might unnecessarily increase the risks of transcapsidation or viral recombination in non vascular tissue (Greene and Alison, 1994). Some authors (Graham et al., 1997) have used phloem specific promoters (as RolC derived from *A. rhizogenes* or Sh, derived from maize) and compared them with the widely used 35S CaMV promoter. Results have shown the these promoters are more specific, limiting their function to the phloem, but as well to neighbouring cells. RolC has a strong activity, which is comparable to that observed with 35S CaMV. These authors performed a resistance test with CP PLRV transformed potatoes, and their results showed that ELISA average levels were reduced, but “resistance“ was comparable to the one observed using the same construct under control of the 35S CaMV promoter.

This study was carried out within the project “Erstellung von Basismaterial bei Winterraps mit Resistenz gegenüber dem Wasserrübenvergilbungsvirus (TuYV, syn. Westlisches Rübenvergilbungsvirus, BWYV) mit verschiedenen gentechnischen und konventionellen Ansätzen, Teilvorhaben Braunschweig” (“Production of basic material in oilseed rape with resistance against *Turnip yellows virus* (TuYV, syn. *Beet western yellows virus*, BWYV) with different genetic techniques and conventional methods, Part Braunschweig”). The research was financed by FNR 97NR041-F. This work was carried out in the Biologische Bundesanstalt für Land- und Forstwirtschaft (BBA), Braunschweig, Institut Pflanzenvirologie, Mikrobiologie und biologische Sicherheit, in the laboratory of Dr. J. Schiemann. The objective was to test resistance in oilseed rape against BWYV. For this purpose different constructs have been made, including the CP, non translatable CP and the replicase gene of the virus. In a first instance, constructs have been transferred to *N. benthamiana* plants, which is a susceptible host of the virus to test for resistance.

Since resistance obtained in transgenic plants transformed with the viral polymerase gene as target seems to be more effective than the one observed by CP, and due to the fact that CP of BWYV was unsuccessful in conferring resistance in lettuce (Gielen et al., 1996), we used the viral polymerase gene of BWYV, encoded by ORF1 and ORF2, to transform *N. benthamiana* plants. At the same time two smaller constructs, which contained the first 400 bp from ORF0 and the last 100 bp of ORF5, in sense or antisense orientation were cloned. Transgenic plants were inoculated with BWYV, with the green peach aphid (*Myzus persicae*) as vector to test for viral resistance under greenhouse conditions.

2. Materials and Methods

2.1. Equipment

Autoclave	Certoclav Cleomat
	Sanoclav Wolf
Automatic film developer	Agfa Curix 60
Camera	Pentax P 30 T
Centrifuges	Centrifuge 5402 Eppendorf
	Biofuge 15 Heraeus
Clean bench	Heraeus Lamin Air
	Ceag Shrip
Electroporator	BIO-RAD Pulse Controller
Electrophoresis	Gibco BRL
	BBA Braunschweig
ELISA Photometer	Molecular devices from MWG Biotec
Growth chamber	Rubarth-Apparate GmbH
Ice machine	Ziegra
Microwave	Toshiba
Micropipettes	Gilson and Eppendorf
pH meter	WTW pH 537
Photoaparat for agarose gels	Camera and transilluminator Kappa-Meßtechnik
	Monitor Panasonic
	Videoprinter 4 P 860 CE Sony
Speedvac	Bachofer Vaccum Concentrator
Thermoblock	Eppendorf 5320
Vortex	Vortex Genie, Scientific Industries
Waterbath	Gesellschaft für Labortechnik (GFL)
	Thermomix BU, B.Braun
	Minitherm, Dinkelberg
Water destillator	Milli Q plus, Millipore

2.2. Greenhouse

The greenhouse used in this study is located in the Biologische Bundesanstalt für Land- und Forstwirtschaft, Braunschweig. It has controlled conditions of temperature and light. For both resistance tests the temperatures were setted at 23/16°C for 18/6 hrs, respectively. During the second resistance test artificial light was added in order to keep the 18 hours light used in the first test.

2.3. Materials

Films for chemioluminescense were obtained from Agfa, positive-charged nylon membrane from Boehringer Mannheim and plastic materials were obtained from Eppendorf, Greiner, Gilson, Roth, Sarstedt and Falcon.
Chemicals were obtained from Biorad, Difco, Duchefa, Eurogentec, Fermentas, Fluka, Gibco, Pharmacia, Riede De Haen, Roth, Serva, Sigma and Boehringer-Mannheim.
The restriction enzymes used in this study were obtained from Boehringer Mannheim, MBI Fermentas and Promega.

2.4. Kits

DIG DNA Labelling and detection kit	Boehringer Mannheim (Cat. N° 1093657)
PCR DIG Probe Synthesis kit	Boehringer Mannheim (Cat. N° 1636090)
DIG Nucleic Acid Detection	Boehringer Mannheim (Cat. N° 1175041)
BCA Protein Assay Reagent kit	Pierce Chemical Company (Cat. N° 500-0002)
Iso-Quick kit	Microprobe (Cat. N° MXT-020-100)
QIAquick kit	QIAGEN (Cat. N° 28304)
QIAEX II kit	QIAGEN (Cat. N° 20021)
NUCLEOBOND PC-kit 500	Machery-Nagel (Cat. N° 740574)
Mikrobank ™	Mast Diagnostika (Cat. N° PL.160)

2.5. Solutions and Media

2.5.1. Media

LB liquid (Sambrook et al., 1989)	10 g/l Trypton 5 g/l yeast extract 10 g/l NaCl	pH 7,0 (NaOH)
LB solid	LB liquid + 15 g/l microagar	
MS (Murashige and Skoog, 1962)	4,6 g/l MS Basal Medium with macro and micro elements and vitamins 20 g/l sacarose	pH 5,7 (KOH)
MS solid	MS liquid + 7 g/l Phytagar	
MS I	MS solid with 2.5 mg/l BAP,	pH 5.8 with KOH
MS II	MS solid with 1 mg/ BAP and 0.1 mg/l NAA,	pH 5.8 with KOH

Antibiotics	Ampicillin	100 g/l H_2O
Carbenicillin	100 g/l H_2O	
	Kanamycin	25 g/l H_2O
	Rifampicin	25 g/l DMSO

2.5.2. Solutions

TE	10mM Tris-HCl, pH 8 1 mM EDTA
Hepes buffer pH 7,0 with KOH	1mM Hepes
TAE	40 mM Tris-acetate pH 7.5 20 mM sodium acetate 1 mM EDTA
Southern I	0.5 M NaOH 1.5 M NaCl
Southern II	1 M Tris pH 7.4 1.5 M NaCl
20 x SSC	3 M NaCl 0.3 M Sodium acetate pH 7.0
DIG-Buffer I	0.1M Malic acid 0.15 M NaCl pH 7.5
DIG-Buffer II	DIG-Buffer I with 2% Blocking reagent (Boehringer Mannheim)
DIG-Buffer III	0.1 M Tris-HCl pH 9.5 0.1 M NaCl
Washing solutions for Northern and Southern blots	2x SSC ; 0.1% SDS 0.5x SSC ; 0.1% SDS 0.1x SSC ; 0.1% SDS
Transfer Buffer for Northern blot	5 X MOPS 0,01 M NaOH

2.6. Plasmids

pFF19G

This is a pUC derived vector which contains 6,000 bp and one replication origin. A β-glucuronidase coding activity, a 2 times enhanced 35S promoter and poly A, both derived from CaMV are as well present. The vector confers ampicillin resistance in bacteria (Timmermann et al., 1990).

pCK GFP S65C

This vector has 4,500 bp, containing a 35S promoter and polyA sequence derived from CaMV, a TL region and the GFP gene (810 bp). The rest of the vector is derived from pUC18, therefore it has a replication origin and confers ampicillin resistance in bacteria. The GFP gene was removed and the remaining vector (~3,700 bp) was used for cloning purposes (Reichel et al., 1996) .

pBin 19

pBin19 (Frisch et al., 1995; Bevan, 1984) was used as a binary vector for *Agrobacterium tumefaciens* mediated transformation of plants. It has 11,777 bp, where the T-DNA region is located between nucleotides 6,043 and 9,421, with a multicloning site located at 6,800. One kanamycin-resistance gene is used as a selectable marker in bacteria and a chimeric nos-nptII-nos gene is located between the T-DNA borders and provides a marker in plant tissues.

2.7. Primers

The different primers synthesized for this study were obtained by Gibco BRL. They corresponded to:

i) ORF1/2 (fragment size 3100 bp)

5'end ORF1 (with NcoI site) (nt 180-194)
5'CCACCATGGATTTCAGAATTGATATTTTTCTTCG3'

3'end ORF2 (with BamHI site) (nt 3,280-3,300)
5'GCGGATCCTTACTCCCTGGATATCTTTTGTGG3'

ii) 5'3'S and 5'3'AS (fragment size 400 bp)

5´end ORF0 (BamHI-BamHI) 300 bp (nt 1-300)
5'GCGGGATCCACAAAAGAAGAAACCAGGAGGGAATCC3'
5'GCGGGATCCAACCAGGCACAAAGCTCTGGTTCGG3'

3'end ORF5 (Xba-PstI) 100bp (nt 5,600-5,692)
5'CGCTCTAGAGTCAAGCCAGAGACATTAAACTGG3'
5'GCCCTGCAGACACCGAAGTGCCGTAGGGAGTTATCC3'

iii) Probe for the 5'end ORF1/2
SV1 (nt 623-642) (fragment size 460 bp)
5'GCAAGGCGAGACAGAAGACG3'
5'CCACCATGGATTTCAGAATTGATATTTTTCTTCG3'

iv) Primers for the 3'end ORF1/2
SV2 (nt 2,731-2,753) (fragment size 369)
5'CGTCCGCAATAGACTTACCATCG3'
5'GCGGATCCTTACTCCCTGGATATCTTTTGTGG3'

v) nptII primers
5'GGTGCCCTGAATGAACTG3'
5'TAGCCAACGCTATGTCCT3'

2.8. Constructs

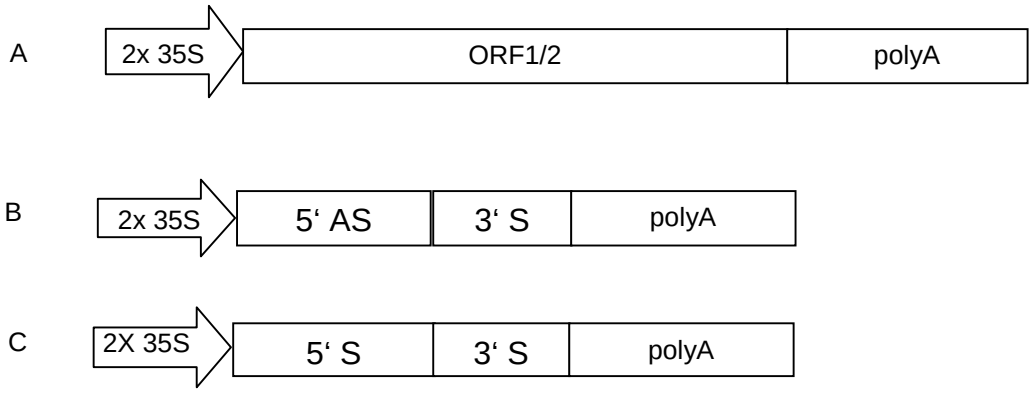


Figure 2 Constructs used for plant transformation. A shows the ORF1/2 construct, which contains the entire open reading frame 1 and 2 (ORF) of the BWYV genome. B and C show the constructs 5‘3‘AS and 5‘3‘S, respectively. 5‘ corresponds to the first 400 bp of the 5‘end of the viral genome inserted in sense (5‘S) or antisense (5‘AS) orientation, while 3‘S represents the most 100 bp end of ORF5. In all cases 2x 35S is the double enhanced promoter and polyA a terminator, both derived from CaMV.

These constructs were synthesized as follows:

i) **pSV ORF1/2**: (Fig 2A) The entire ORF1 and ORF2 from BWYV were synthesized by PCR using the primers indicated in section 2.7 and the full length genome of BWYV as template. The PCR was carried out under standard conditions. The resulting PCR product (3,100 bp) was loaded on a 0.8% agarose gel, the fragment was sliced out and further purified using a QIAEX II agarose gel extraction kit (#28304). The purified fragment was digested with NcoI and BamHI at 37°C, the enzymes were eliminated and the PCR product was ligated into a modified pCK GFP S56C vector. To amplify the plasmid DNA, it was electroporated in *E. coli* and further purified as indicated in 2.11.2. The plasmid was digested with HindIII resulting in a 4,100 bp cassette, which contained the two times enhanced 35S CaMV promoter, the ORF1/2 insert and the polyA sequence. The complete cassette was inserted in the binary vector pBin19, using the HindIII site and further transferred to *A. tumefaciens* by electroporation.

ii) **pSV 5’3’AS and pSV 5’3’S** (Fig 2, B and C, respectively). For the cloning of these fragments the full length clone of BWYV was used as template and the corresponding primers indicated in section 2.7. The resulting 100 bp fragment of the 3’ end was extracted from an agarose gel, purified with a QIAEX II agarose gel extraction kit (# 28304) and digested with XbaI and PstI. Finally it was inserted in the pFF19G vector, between the 35S CaMV promoter and polyA sequence, resulting in

the vector pFF19 3' This vector was further digested with BamHI, and then the 5' end fragment (400 bp) was ligated, which, due to the fact that both ends, i.e. 5' and 3' end had a BamHI site, it could be inserted in sense or antisense orientation, giving rise to either 5'3'S pFF19 or 5'3'AS pFF19. The vectors were digested with HindIII and NcoI resulting in a 1,400 bp fragment, which contained the 35S CaMV promoter, the corresponding fragment, and the polyA sequence, derived from CaMV. The fragment was purified from an agarose gel and ligated to the binary vector pBin 19. These vectors were transferred to *A. tumefaciens* by electroporation (see 2.10.2).

2.9. General Methods

2.9.1. Ligation and digestions with restriction enzymes

The T4 ligase from Promega™ was used following the manufacturers instructions. Previous to ligation, DNA fragments were dephosphorylated with CIP (calf intestine alkaline phosphatase). Usually a 1:3 ratio of plasmid:DNA fragment was used. A negative control was included in each case, which consisted of the dephosphorylated plasmid alone, this gives an estimate of the religation rate of the plasmid.
Enzyme restriction digestions were carried out following the protocol described by Sambrook et al. (1989).

2.9.2. Agarose gel electrophoresis

DNA was identified and separated using 0.7 to 1.5% agarose gels in TAE buffer containing ethidium bromide (40 µl/l). The appropriate molecular weight marker(s) was used in each case. The gel was run at 4 V/cm and examined by UV light at 254 nm, using a transilluminator. Gels were photographed to record results.

2.9.3. DNA extraction from agarose gels

DNA samples were run on agarose gels and examined under special UV light (360 nm). The DNA fragments of interest were sliced out from agarose gels and treated according to the QIAEX II agarose gel extraction kit (# 28304), following the manufactures instructions.

2.9.4. PCR
2.9.4.1. Standard PCR mixture

Unless otherwise indicated the standard mixture used for PCR corresponded to the following:

PCR mixture	2 µl 10 x PCR buffer
	1 µl NTPs (10 mM)
	0,2 µl Primer 5´(10 µM/µl)
	0,2 µl Primer 3´
	0,2 µl Takara polymerase (0.5 U/µl)
	1 µl template DNA (ca. 10 ng)
	15,4 µl bidest. water
	Total volume 20 µl

The PCR was carried out under the following conditions
3 min. delay at 94°C
1 min. denaturing at 94°C
1 min. annealing at 55°C
1 min. elongation at 72°C
for 30 cycles. An aliqout of the PCR products was analyzed by agarose gel electrophoresis.

2.9.4.2. PCR product purification

PCR products were purified using a QIAGEN kit (Catalogue N° 28304), following the manufacturers instructions.

2.9.4.3. Detection of transgenic plants by PCR

PCR can provide a useful tool to demonstrate the presence of specific DNA sequences within the genome; however, it is important to remember that more information about the number of copies and arrangement of the foreign DNA in the plant genome can be obtained by Southern blot analysis.
The detection of the corresponding transgene fragment in the genomic DNA of the transformed plants was analyzed by PCR. For this purpose genomic DNA isolated from the transgenic plants (see section 2.13.1) was used as a template in a standard PCR mixture using Takara Taq Polymerase™. The PCR was carried out for 1 min. at 94°C, 1 min. at 55°C and 2 min. at 72°C during 30 cycles. The primers used in each case were the same as those described above (as described in section 2.7).
The PCR products were analyzed in a 1% agarose gel electrophoresis. In each case two negative controls were included, which corresponded to i) DNA isolated from a non transformed *N. benthamiana* and ii) water control, which contained the PCR mixture without DNA. As positive control the corresponding plasmid DNA was used as template.

In order to confirm that the products obtained by PCR corresponded to the construct under study, they were further analyzed by Southern blot using a specific DIG labeled probe for each construct.

A PCR for the presence of the nptII gene was carried out. For this purpose an aliquot of the purified genomic DNA was added in a standard PCR mixture containing the nptII primers (section 2.7). The following conditions for PCR were used: 1 min. at 94°C, 1 min. at 60°C and 2 min. at 72°C, during 30 cycles. In each case a negative and positive control as mentioned above were included.

2.9.4.4. Synthesis of DIG-labeled probes

The DIG system uses digoxygenin, a steroid hapten in the form of DIG-11-dUTP, to label DNA, RNA or oligonucleotides. Probes can be produced by different methods. In this case probes were produced by PCR.
Four different DIG labeled probes were synthesized in this study, which corresponded to the 5'end and the 3'end of ORF1/2 insert, the 5'end of the 5'3'AS and 5'3'S constructs and a nptII probe.
The DIG labelling was performed by PCR using a kit from Boehringer (PCR DIG Probe Synthesis Kit, Cat. #1636090), following the manufacturers instructions. The primers used for the synthesis of the different probes are described above (see section 2.7).
The Takara polymerase was used instead of the one provided by the kit. The standard PCR mixture used corresponded to:

5 µl 10x PCR-Buffer
10 µl PCR DIG Mix 5x
5 µl Primer 5'(10 µM/µl)
5 µl Primer 3'(10 µM/µl)
0.5 µl Taq-Pol (0,5 U/µl)
1 µl template DNA (ca. 10 ng)
23,5 µl bidest. water

The PCR was carried out under the following conditions 3 min. delay at 94°C, 1 min. denaturing at 94°C, 1 min. annealing at 55°C and 2 min. elongation at 72°C, for 35 cycles. An aliquot of the probe was run on an 1% agarose gel in order to check the size of the fragment obtained. It must be kept in mind that the product seems bigger than the non-labelled control, since it contains DIG-UTP. The concentration of the probe was determined by making serial dilutions of the DIG-labeled probe as indicated by the manufacture. Aliquots of the probe were kept at -20°C.

2.10. Competent bacteria for electroporation

For the transformation of bacteria with foreign plasmids it is necessary to have bacteria which can easily uptake the vector by electroporation. Therefore competent bacteria were produced by the following method.
1l of LB medium was inoculated with 10 ml of an overnight culture of *E. coli* (DH5α) and allowed to grow at 37°C to an OD_{600} of 0.5 – 0.8. At this time the culture was left on ice for 15 minutes and then centrifuged at 4,000 rpm for 10 minutes at 4°C. The pellet was washed twice with ice cold sterile water, resuspended and centrifuged. Finally it was washed with 20 ml of ice cold glycerin (10%). The pellet was then resuspended in 2-3 ml of 10% glycerin, aliquoted, immediately left on liquid nitrogen and stored at -70°C for further use.

In order to obtain competent *A. tumefaciens* (EHA 101), the same basic protocol was used, with the following modifications
i) bacteria were grown at 28°C in a modified LB medium
ii) washed with 1mM HEPES pH 7.0 instead of water.

2.10.1. Electroporation of *E. coli*

1µl of the plasmid (c = 1 µg/µl) was incubated with 40 µl of competent bacteria, transferred to an ice-cold cuvette (BioRad) and electroporated under the following conditions:
Resistance 200 Ω
Capacitance extender 125 µF
Set Volts 2.5 kV
Capacitance 25 µF.

After electroporation (ca. 4,5 ms) 1 ml of SOC medium (i.e. LB plus 20 mM glucose) was added and *E. coli* was allowed to grow at 37°C for 30 minutes, respectively. After this time different aliquots (10-100 µl) of bacteria were plated on LB medium containing the corresponding antibiotics for selection. The plates were incubated during one day at 37°C. In order to have an idea of religation of the plasmid itself, a negative control was done, which consisted of bacteria transformed only with the plasmid, which had been treated with T4 ligase in absence of the insert.

2.10.2 Electroporation of *A. tumefaciens*

The procedure for electroporation of *A. tumefaciens* was similar as the one used for *E. coli.* After electroporation bacteria were kept at 28°C for 4 hours and the aliquots

were plated and incubated for two days at 28°C. Besides the antibiotic for selecting the positive colonies, plates contained 50 mg/l of rifampicin.

2.11. Plasmid isolation from bacteria

Once the bacteria were electroporated with the plasmid of interest, they were allowed to grow overnight and plasmid DNA was purified. According to the amounts and purity of the plasmid DNA required, two different methods were used. Minipreps are useful to follow the cloning strategy, while midipreps allow to obtain large amounts of DNA, once it has been tested as correct.

2.11.1. Minipreps for DNA isolation

A single bacterial colony was picked up with a toothpick, transferred to 3 ml of LB medium containing the appropriate antibiotics and incubated overnight at 37°C. 1.5 ml of this culture was centrifuged at 14,000 rpm for 30 s and the pellet was resuspended in 200 µl of Solution I (10 mM EDTA, 50 mM Tris, pH 8) which contained RNAse and left at room temperature for 5 minutes. Solution II was then added (0.2 N NaOH, 1% SDS) and the tubes were carefully mixed, after additional 5 minutes at room temperature 200 µl of ice cold solution III (3M KAc, pH 4.8) was added, carefully mixed and centrifuged for 10 minutes at 14,000 rpm at 4°C. The supernatant was transferred to a fresh tube and DNA was further precipitated with isopropanol. The sample was left for 10 minutes on ice it was centrifuged for 15 minutes at 14,000 rpm at 4°C. The pellet was washed with 70% ethanol, centrifuged, allowed to dry in a speedvac and finally resuspended in 30 µl TE buffer.
For *A. tumefaciens* the same basic protocol was followed, only that the bacteria were grown at 28°C in modified LB medium and a phenol-chloroform extraction was carried out before isopropanol precipitation.

2.11.2. Midipreps for DNA isolation

To obtain larger amounts of plasmid DNA 50 ml of culture of the bacterial strain carrying the plasmid of interest were grown up to an OD_{600} of ~0.6-0.8. The culture was treated under the same basis as described in 2.11.1., but in this case NUCLEOBOND PC-Kit 500 kit was used (Machery-Nagel # 740574). The pellet was resuspended in 200-300 µl TE, pH 8.

2.12. Plant material and transformation

2.12.1. Plant material

The *Nicotiana benthamiana* plants used to obtain the starting leaf material required for plant transformation were grown either in the greenhouse or in a growth chamber with a 14 hr light/ 10 hr dark cycle at 25°C.

2.12.2. Plant transformation

Plants were transformed with *Agrobacterium tumefaciens*, containing either pSV ORF1/2, pSV 5´3´AS, pSV 5´3´S or pBin19, using the leaf disc method (Horsch et al., 1985).
Nicotiana benthamiana leaves obtained from 4 to 6 week old plants grown in the greenhouse were sterilized for 20 minutes with a 1.2% sodium hypochlorite solution and thoroughly washed with sterile water, *in vitro* plants could be used directly. The leaves, from which the midrib and the edges had been removed were cut into discs of 0.5 cm x 0.5 cm.
One day before the inoculation, ca. 10 ml of LB mod culture of the LBA4404 strain harboring the different DNA constructs was set up. Km and Rif were added and it was allowed to incubate overnight at 28°C, with shaking. On the next day the OD_{600} was measured, and adjusted to ca. 0.8 to 1.
Leaf discs were co-cultivated with the bacteria in petri dishes containing 10 ml of MS liquid medium and 0,2 ml of the overnight grown *A. tumefaciens* suspension. They were incubated in the dark during 2 days at 26/16 °C for 16/8 hours, respectively. After this time leaf discs were removed, washed 5 times with sterile water, dried on filter paper and carefully transferred to MS solid medium which contained kanamycin (100 mg/l) as selector for transgenic plants and beta-bactyl (Ticarcillyn 300 mg/l), in order to eliminate the bacteria and hormones to induce callus formation (1 mg/l BAP). Leaf discs were incubated at 26/16°C for 16/8 hours of day/night, respectively. Petri dishes were checked daily for presence of bacteria or possible contamination. Leaves were changed every 2 to 3 weeks to fresh MS medium. After ca. 4 weeks resistant calli could be obtained, these were further transferred to new media.
Resistant calli were induced to regenerate shoots on MS medium, by adding hormones (0.1 mg/l NAA and 1 mg/l BAP). The MS medium contained 100 mg/l kanamycin and 300 mg/l Ticarcilin. They same temperature and light conditions described above were ed. Once the calli became shoots, usually at 8-10 weeks after transformation, they were transferred to MS rooting medium which contained BAP (0.1 mg/l), besides the corresponding antibiotics. Shoots had developed roots after ca. 16 weeks.

Once the plantlets had developed roots and had three to four pairs of leaves, they were transferred in to pots in the greenhouse. For this purpose, the agar was carefully removed from the roots with warm water and plants were planted in pots containing soil (Floraton 2, Firm Florograd Product). At this time they were well watered and covered with a plastic bag. After one week, plastics bags were opened to allow a better air exchange. They were completely removed after keeping plants for ca. 2 to 3 weeks in the greenhouse. Plants were kept under day temperature of 24°C and 18°C during the night.

2.13. NPTII ELISA (Engvall and Perlmann, 1974)

To measure the expression of the nptII gene from the transformed plants an NPTII ELISA was done following the kit protocol (5 Prime -3 Prime Inc™ #5307-610101). The starting material corresponded to 100 mg of fresh leaf which was ground in 3 ml of sample buffer (PBS Tween 20). Due to the fact that transformed cells can contain a wide range of NPTII protein, dilutions were made to assure that their concentration was in the linear range of the kit. For this purpose the protein concentration of each sample was adjusted to 400 ug/ml by using the BCA Protein Assay Kit ™ (# 23255) Pierce (see below). A standard NPTII curve was included for each NPTII ELISA assay.

Protein concentration was estimated using the BCA Protein Assay Kit ™ (# 23255), which is based on the Biuret method. 100 mg of leaves were ground in 3 ml ELISA sample buffer (PBS + Tween 20), and 50 µl of diluted samples (1/20) were added to 1 ml of reactant solution. Samples were incubated for 30 minutes at 37°C and color development was measured at 562 nm. A standard BSA curve (0 to 2,000 µg/ml) was made in parallel for each assay. Protein concentration was interpolated from the plotted data of the BSA concentration vs OD_{562} obtained.

2.14. Genomic DNA

The existing methods for the isolation of genomic DNA vary enormously, having the quantity and quality of DNA required an important factor on the decision of the method choosen.

2.14.1. DNA extraction for PCR

Plant genomic DNA was extracted according to the method of Hart (1985). Briefly, 100 mg of leaf material was ground in extraction buffer (100 mM Tris-HCl, pH 8; 50 mM Na_2-EDTA, 500 mM NaCl and 1.25% SDS, plus 40 mg sodium bisulfite freshly added per each 10 ml of buffer) and incubated at 60°C for 45 min. Samples were

allowed to cool at room temperature. This was followed by a chlorform-isoamylalcohol (24:1) extraction and centrifugation at 10,000 rpm for 10 min. The aqueous phase was separated and treated with RNase A for 20 min at 37°C. DNA was precipitated by addition of 0.7 vol. of isopropanol. Samples were centrifuged at 14,000 rpm for 10 min. The pellet was washed once with 76% ethanol, 0.2 M NaAc for 30 minutes and a short wash with 76% ethanol, 10 mM NH_4Ac was followed. The pellet was resuspended in 100 µl TE. An aliqout of the purified genomic DNA was loaded on an 0.7% agarose gel to have a rough estimation of the quantity obtained.

2.14.2. DNA concentration

In order to estimate the DNA concentration, 1 µl of purified DNA was diluted in 49 µl of bidest water and the optical density (OD) was measured in a range from 220-320 nm using a spectrofotometer. An OD of 1.0 at 260 nm equals a dsDNA concentration of 50 µg/ml (Sambrook et al., 1989).

2.15. Total RNA from transgenic plants

2.15.1. RNA isolation

In order to analyse the expression of mRNA in the transformed plants by Northern blot total RNA was extracted. It is important that all materials used during this procedure are RNAse-free, therefore they must be previously autoclaved. For the extraction of RNA, 100 mg of fresh leaf material was ground in liquid nitrogen and treated as indicated in the protocol of RNA easy pure™ according to the manufactures instructions. The pellet was resuspended in 20 µl of DEPC-treated water and incubated at 55°C for 10 minutes to dissolve the RNA. Samples were kept at -70°C until analysed by Northern blot.

2.15.2. RNA concentration

The RNA concentration was measured photometrically. 1 µl of the RNA sample was diluted in 500 µl of DEPC-treated water. The solution was measured at 260 and 280 nm, using water as blank. An OD_{260} of 1 corresponded to a RNA concentration of 40 µg/ml. The ratio 260/280 gives an indication of the purity of the RNA, this value should be between 1.7 and 2.

2.15.3. Northern blot

All solutions used for Northern blotting were previously treated with DEPC, for at least 4 hours and then autoclaved for 20 minutes.
Isolated RNA (20 µg/µl) from the different transgenic lines was incubated with gel buffer (MOPS), formamide and formaldehyde for 15 minutes at 55°C. Sample buffer was then added. A RNA Molecular Weight Marker II, (Boehringer Mannheim) was treated under the same conditions. Samples were loaded on a 0.7% agarose formaldehyde gel. The gel was run in 1 x MOPS buffer at 4 V/cm during 3 to 5 hours. After this time, the marker was cut out, stained with ethidium bromide and photographed. The gel containing the samples was washed with 5 x SSC for 5 minutes and then RNA was transferred to a nylon membrane, using 5 x SSC-0.01 M NaOH as transfer buffer. The transfer was allowed to proceed for at least four hours or overnight. The membrane was removed, marked on one edge and washed with 5 x SSC and finally crosslinked with UV light for one minute on each side.

2.16 DIG detection of blots

2.16.1. Prehybridization and hybridization of membranes

The membranes containing either the fixed RNA were prehybridized, in order to block the attachment of the probe to non specific nucleic acid-binding sites. For this purpose, the nylon membranes were placed in a plastic bag, filled with 10 ml of Ultrahyb™ solution per 100 cm^2, further sealed and incubated in a waterbath at 48°C for at least one hour.

The corresponding DIG labeled probe to be used was denaturated at 100°C for 10 minutes and allowed to cool on ice for further 10 min. The probe was added to a Ultrahyb™ solution so as to reach a final concentration of 1 pg/ml of a 300 nt probe.
After prehybridizing, the solution was removed, poured into a Falcon tube and kept at -20°C, since it can be re-used in other assays. The hybridizing solution, pre-warmed at ca. 68°C was carefully poured into the plastic bag which was further sealed. The membranes were incubated for 16 to 20 hours in a waterbath at 48°C. After this time the hybridizing solution was removed, stored at -20°C and the membrane was quickly placed in a tray containing 2 x SSC + 0.1% SDS for 5 minutes, to remove the excess of probe.
Washing of the membrane was performed by adding twice 2 x SSC, 0.1% SDS for 15 minutes at room temperature, 0.5 x SSC, 0.1 SDS at 68°C for 15 minutes and finally once 0.1 x SSC, 0.1% SDS for 15 minutes at 68°C. As mentioned before, all solutions used for Northern blot analysis were previously treated with DEPC.

2.16.2. Chemiluminescent detection

To detect the bands that had hybridized with the DIG-labeled probe, an alkaline phosphatase-conjugated antidigoxigenin antibody and a chemiluminescent substrate were employed. Chemiluminescent substrates can be visualised by exposure to X-ray films.
The membrane was firstly equilibrated in DIG-Buffer I for 3 minutes. The blocking step was done by gently agitating the membrane in 20 ml of 2% blocking reagent (Boehringer Mannheim) for at least one hour. The blot was incubated with the anti-DIG alkaline phosphatase antibody for 30 minutes (1/10,000 in DIG-buffer II) at room temperature and thoroughly washed 3 times with DIG-buffer I. In order to activate the enzyme, the membrane was equilibrated in DIG-buffer III for 5 min. and then removed and placed face-up on a plastic film, covered with substrate solution CSPD (disodium 3-(4-methoxyspirol{1,2-dioxethane-3,2´-(5´-chloro)tricyclo[3,3,1,1] decan}-4-yl)phenyl phosphate) for 5 min. at room temperature. The excess of this solution was gently removed using a paper towel and the membrane was incubated for 10 to 15 min. at 37°C. Finally it was exposed to an X-ray film for different times and then the film was developed.

2.16.3. Reproving of blots

To reprove Northern blots, membranes were washed with a 0.1% SDS solution (prepared with DEPC-treated water) at ca. 90°C for 10 minutes. The membranes were equilibrated in 2 x SSC, and then the prehybridizing step was followed.

2.17. Segregation of plants on kanamycin

2.17.1. Seed sterilisation

Seeds obtained from self fertilised transgenic *N. benthamiana* plants were surface sterilized with 70% ethanol for 3 minutes and thoroughly washed five times with sterile water. After the last wash, seeds were layed on filter paper and allowed to germinate at 26/16°C, with a day/night period of 16/8 hours respectively during one week.

2.17.2. Segregation of transgenic lines on kanamycin

Germinated seeds (ca. 25 per plate) from the different transgenic lines were placed on MS medium containing Km (100 mg/l), 4 plates per line. They were placed in a growth chamber at 18/6 h day/night at 25/18°C for 3 to 4 weeks. The ratio of sensitive and resistant plantlets was determined and a x^2 test was carried out. The

same was done with some transgenic lines which were tested at higher concentrations of kanamycin (i.e. 200 and 300 mg/l).

2.18. Greenhouse resistance tests

2.18.1. Preparation of transgenic plants for the greenhouse resistance test

In order to carry out the resistance test against BWYV, seeds (ca. 120) from each line to be assayed were surface sterilized and placed on filter paper and after one week they were transferred on selection medium (200 mg/l kanamycin) for further 2 to 3 weeks (see 2.16). Once they had developed their first leaves ca. 80 to 100 plantlets per line were transferred to the greenhouse. Plantlets of each line to be tested were separated in two sets of ca. 40 each. They were kept in the greenhouse for at least 5 days at 24°C for 16 hours, dropping the temperature to 18°C during the night.

2.18.2. Greenhouse resistance tests

To test for resistance against BWYV, 5 transgenic *N. benthamiana* lines from each construct were selected. As susceptible controls for virus infection, two lines of *N. benthamiana* plants transformed with the pBin19 plasmid alone were used (vector control), as well as untransformed *N. benthamiana* plants.
When plantlets had developed their first 4 to 5 fully expanded leaves 20 of them, which had similar size were infected with BWYV, by means of *Myzus persicae* as a vector; while the other set containing 20 plants was left as healthy control. Aphids were allowed to feed from the plants for 3 days, after this time they were eliminated by applying an insecticide which was also applied to the healthy controls. Plants were allowed to develop for further 8 weeks under the greenhouse conditions
Leaf samples from each infected plant of all lines tested were taken at 4, 6 and 8 wpi. to perform BWYV-ELISA analyses. Samples of three healthy randomly selected plants from each line were collected at the same time, to assure that no virus was present in the healthy control. The height of each plant (infected and healthy) was measured at the same sampling periods, and the final weight was determined for each plant at the end of the experiment (8 wpi).

2.18.2. Aphids and Virus Propagation

The green peach aphid (*Myzus persicae*) was maintained in virus free colonies on individually caged pepper plants (*Capsicum annuum*) in a growth chamber at 20°C with a 18 hr photoperiod.

Oilseed rape plants (*Brassica napus*) to be used as virus sources were inoculated with viruliferous aphids for 3 days, after this time aphids were eliminated with an insecticide. At three weeks after infection, leaf material was collected from each plant, ground in sample buffer (PBS-Tween 20) and tested by BWYV ELISA. Only those plants which had OD_{405} values equal to or higher than 1 were kept as virus source plants.

2.18.3. TAS BWYV ELISA

The presence of viral antigen in transgenic plants was analyzed by standard TAS ELISA. To perform TAS ELISA, 100 mg of leaf material was homogenised in sample buffer (PBS, Tween 20, and NaN_3, pH 7.4). Polyclonal antiserum raised the coat protein of BWYV isolated from *Physalis floridana* was used as the primary antibody. The microtiter plates (Greiner) were coated with IgG-BWYV (1/500 in coating buffer, Na_2CO_3, $NaHCO_3$, NaN_3, pH 9.6) for 4 hours and after washing three times with PBS-Tween, samples were incubated overnight at 4°C. After washing the plate three times with PBS-Tween and blocked with defatted milk (30 minutes, 37°C), it was incubated with Mab5G4 (diluted 1/1000 in sample buffer) and then by rabbit anti-mouse antibody (1/1000, diluted in PBS) alkaline phosphatase conjugated. As substrate p-nitrophenylphosphate was used. After allowing 30 minutes for colour development, the plate was read at 405 nm. Each microtiter plate had the corresponding negative controls (uninoculated transformed *N. benthamiana* and non transformed *N. benthamiana* plant extract).

2.18.4. Resistance test of transgenic plants not selected on kanamycin

Two transgenic lines containing the ORF1/2 construct, SV 112 and SV 117, were analysed for their response against BWYV, without previous selection on kanamycin. In this case, seeds from each line were directly planted into soil in the greenhouse. After germination they were separated in two sets of 10 plants each. Genomic DNA was extracted from leaves of each plant and further analysed for the presence of the viral insert by PCR. These plants were assayed in parallel to the second resistance test of the ORF1/2 lines. Ten plants of each line were challenged with BWYV by transferring 5 to 7 green peach aphids on new leaves. As done in the other greenhouse resistance tests, 10 plants of each line were kept as healthy controls. Aphids were allowed to feed for 3-4 days and at this time they were eliminated with an insecticide, which was also applied to the respective healthy controls. Plants were maintained in the greenhouse for further 8 weeks. Leaf samples from inoculated and healthy plants were taken at 4, 6 and 8 wpi and analysed by BWYV ELISA as described in section 2.18.3. The height of each plant was measured at the same sampling times and its final weight was estimated at the end of the experiment.

3. RESULTS

The objective of this study was to search for resistance against *Beet western yellows virus* (BWYV). For this purpose *Nicotiana benthamiana* plants were transformed by the *A. tumefaciens* leaf disc method. The viral replicase gene, encoded by ORF1 and ORF2 from the viral genome, was used as target to generate transgenic plants. In paralell two smaller non translatable fragments, containing the 5' end and the 3' end of the viral genome were cloned, transferred to plants and tested for the possibility of conferring resistance against BWYV.

3.1. TRANSGENIC PLANTS

Genetic engineering of plants may be used to modify the expression of genes already present or to introduce new genes which can improve their usefulness. The most common method for the introduction of new genes into plants is based on the natural DNA transfer capacity of *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes.* Vectors for plant transformation may be synthesized by replacing parts of the T-DNA with the DNA of interest to be introduced into the plant.

3.1.1. Construct pSV ORF1/2

The ORF1 and ORF2 of BWYV consist of 3,100 nt and encode for 937 amino acids (Fig. 1). A DNA clone encoding this region was synthesized using a 19 bp primer, at the 180–194 region and a 20 bp primer at region 3,280-3,300, containing HindIII and NcoI sites (see Materials and Methods). The full length clone of BWYV was used as a template. Under the PCR conditions described in Materials and Methods, a product of the expected size (3,100 bp) was obtained, which was loaded on an agarose gel, excised, purified and digested with HindIII and NcoI. This fragment was ligated into the pFF19G plasmid, in the HindIII and NcoI sites, between the double enhanced 35S CaMV promoter and a polyA sequence, giving raise to plasmid pFFORF1/2. The cloned vector was electroporated into *E. coli*, plasmid DNA was isolated from a positive colony and further digested with different restriction enzymes. The fragment (ca. 4,100 bp) was purified using a gel extraction kit, digested with HindIII and ligated into the binary vector pBin19, giving raise to the vector pSV ORF1/2. The purified plasmid was electroporated to *A. tumefaciens*, colonies were picked and grown in liquid LBmod medium overnight, containing the respective antibiotics (Rif and Km). Plasmid DNA was purified and digested with several restriction enzymes, positive colonies were identified and one clone was selected to be used for plant transformation.

3.1.2. Constructs pSV 5'3'AS and pSV 5'3'S

These fragments were synthesized by copying the first 400 bp of the 5' end of the viral genome and the last 100 bp of the 3’ end of the ORF5 of BWYV (Fig. 1). Two different pairs of primers were designed, and the full length clone of the virus was used as template for amplification by PCR. The 5’ end fragment was synthesized using both primers with BamHI ends, while the primers used for the synthesis of the 3’ end of ORF5 had PstI and Xba sites. The 3’ end was first synthesized by PCR, the size was checked by gel electrophoresis. The PCR product purified, digested with the adequate restriction enzymes and inserted into the pFF19G vector, at the Pst and Xba sites. The vector pFF19-3’ was electroporated into *E. coli* under the conditions described in Materials and Methods. Plasmid DNA was purified from a positive colony. The plasmid was digested with BamHI, and the purified 5’ end PCR product was inserted. This fragment could be inserted either in sense or antisense orientation, giving rise to pFF5’3’S or pFF5’3’AS, respectively. After electroporating *E. coli* with these vectors plasmid DNA was extracted from minipreps (see 2.11.1.) and the DNA was digested either with XbaI or EcoRI in order to differentiate bacteria containing the sense or antisense viral sequence, since both restriction sites are present in the 5’ end fragment.

Once the colonies were defined as 5’3’AS or 5’3’S, plasmid DNA was purified, digested with HindIII and EcoRI (partial digestion) and inserted into the binary vector pBin19. In each case the 35S promoter and a polyA sequence, both derived from CaMV, were used. The plasmid was electroporated into *A. tumefaciens*.
The vector control plants were transformed with the binary vector itself (pBin19).

3.1.3. Plant transformation

To test if a given genomic sequence of a pathogen can confer resistance usually model plants are first transformed, before using a specific crop.

A model plant species, for use in such studies, can be defined as one that can be efficiently and simply transformed with foreign DNA. Furthermore, the transformed cells or tissues must be able to regenerate and produce fertile mature plants that produce transgenic seeds.

N. tabacum and *N*. *benthamiana* are commonly employed as model plants to study pathogen derived resistance (PDR). The most efficient and technically simple method of transformation is to infect leaf explants with *A. tumefaciens*. The method is based on the fact that besides the border repeats, none of the T-DNA sequences is required for transfer and integration. Therefore the T-DNA genes can be replaced by any other DNA of interest, which will be transferred to the plant genome. Since transgenic

plants are phenotypically indistinguishable from untransformed plants, markers have been developed (i.e. antibiotic resistance). Two types of Ti plasmid-derived vectors can be distinguished: i) *cis* systems or cointegrated vectors in which new genes are introduced via homologous recombination into a non-oncogenic Ti plasmid and ii) *trans* or binary systems in which new genes are cloned into a plasmid containing a non-oncogenic T-DNA, which is subsequently introduced into an *Agrobacterium* strain harbouring a Ti plasmid with an intact *vir* region, but lacking the T-DNA region. Following inoculation the leaf explants will regenerate transgenic plantlets. These transgenic plants provide enough leaf material to carry out the different molecular characterisation analyses. It is possible to infect tobacco with different plant viruses, however it is not a host for BWYV. Therefore *N. benthamiana*, which has the same advantages as *N. tabacum* was used for transformation in this study, since it can be infected with BWYV by means of *M. persicae*.

Before each transformation event of *N. benthamiana* plasmid DNA was extracted from the bacterium culture and digested with the appropriate restriction enzymes in order to check for the presence of the viral sequence.

N. benthamiana plants transformed by the disarmed *Agrobacterium tumefaciens* strain LBA 4404 harboring either the vector (pBin19) or the vector containing the different viral sequences cloned (ORF1/2, 5’3’AS and 5’3’S) were generated. Using the standard transformation protocol 3 to 4 months were necessary to allow development of rooted plantlets that could tolerate transfer into soil.

During the different transformation events, it could be observed that some plantlets showed a “glass like” aspect in their leaves. This was further eliminated in most cases by reducing to one tenth the recommended nitrogen level in the MS medium. Some plantlets were unable to develop roots. Plants that showed an unusual phenotype, i.e. leaf curling, glass like aspect or no development of roots were discarded. At least 120 *N. benthamiana* plantlets that could grow at 100 mg/l of Km were produced.

As control for kanamycin selection non-transformed *N. benthamiana* leaf discs were included on media containing this antibiotic. These leaves did not develop further and after ca. 2 weeks they turned white. To check that the MS medium was adequate for the development of plantlets, non inoculated leaves of *N. benthamiana* were placed on MS plates without any antibiotic. After ca. 12-14 weeks plantlets had developed.

3.1.4. Selection of transformants

Transformants containing one of the three different viral constructs or the T-DNA of the binary vector pBin19 were selected by performing PCR amplification of the genomic DNA and/or by an NPTII ELISA assay of leaves of kanamycin resistant plants.

3.1.4.1. NPTII ELISA

Before transferring the Km resistant plantlets to the greenhouse, they were assayed by NPTII ELISA. The product of the nptII gene should be expressed in transgenic plants growing on Km selection media. Quantitative ELISA of NPTII protein was used to determine the activity of nptII gene in leaves of transformed *N. benthamiana* plants, which was driven by the double enhanced 35S CaMV promoter.

Due to the difference of expression of proteins in plants, leaf extracts were previously adjusted to a protein concentration of 400 mg/ml. Aliquots of ground leaves were incubated overnight with the primary antibody and the ELISA was performed on the following day. In each plate a negative control (leaves from non-transformed *N. benthamiana*) and a standard NPTII curve were included.

The levels of NPTII expression determined for the different transgenic plantlets tested varied between 0.4 to 4.3 ng/mg protein (Table 3). Plants that had NPTII expression levels below 0.3 ng/mg protein were considered negative. About 90% of the total plantlets assayed were positive.

3.1.4.2. Amplification of the viral sequences or nptII gene by PCR

PCR is one of the mostly used techniques for screening material for the presence or absence of transgenic sequences. However due to the extreme sensitivity of this method care must always be taken to minimise contamination or false positive results. Special attention in the design of the primers, size of the PCR product (i.e. less than 1,000 bp) and “logistics” (how and where the work is carried out) can help to minimise these risks (Register, 1997).

PCR was carried out either from the viral sequence and/or the nptII gene of regenerated plantlets. Genomic DNA was extracted from small leaves of the Km resistant plantlets, as described in Materials and Methods. Care was taken to avoid possible contamination with *A. tumefaciens*. The primers used for PCR were specific to sequences of the viral genome inserted in the vector, which were absent in plants transformed with the plasmid vector alone or wild-type plants.

3.1.4.2.1. PCR of ORF1/2

To test for positive plants containing the ORF1/2 transgene, two sets of primers were synthesized. The first pair was designed for the 5’ end, which was synthesized at the region 630 giving rise to a 470 bp product and another primer pair was synthesized for the 3’ end, at the 2,750 region producing a fragment of 370 bp.

Genomic DNA was extracted from plants which were in the greenhouse for ca. 6-8 weeks. In each assay genomic DNA of a nontransformed plant was included. Most plantlets were tested for the 5’ end of the complete ORF1/2 fragment only when results were doubtful, the 3’ end of the ORF1/2 was amplified to confirm the presence or absence of the viral fragment. Under the PCR conditions described in Materials and Methods (section 2.9.4.3.) almost all plantlets analysed were positive. The PCR products of some of these lines are shown in Fig. 3.

As seen in Fig. 3, most lines tested were positive. In this example only two lines were negative (SV 89 and SV 119, lanes 5 and 15, respectively). Both plants tested positive by NPTII ELISA, therefore they were transferred into the greenhouse. However, when the genomic DNA was amplified no band was observed. The negative controls included in each PCR assay corresponded to genomic DNA from untransformed *N. benthamiana* and the water control of which in no case bands could be detected (lanes 8, 18, 9 and 19 in Fig. 3).

3.1.4.2.2. PCR of 5’3’S or 5’3’AS

Due to the similarity of the 5’3’S and 5’3’AS constructs, plantlets which contained either of these inserts were analysed together. This transgene was detected in *N. benthamiana* plants by PCR, with the same primers used for its synthesis (see Materials and Methods). In all cases the 5’ end of this construct was amplified, due to its larger size (400 bp) compared to the 3’ end segment (100 bp). Almost all plantlets tested were positive by this method. No product was observed when amplifying genomic DNA from non transformed plants. An example of the PCR products from some plants tested is shown in Fig. 4.

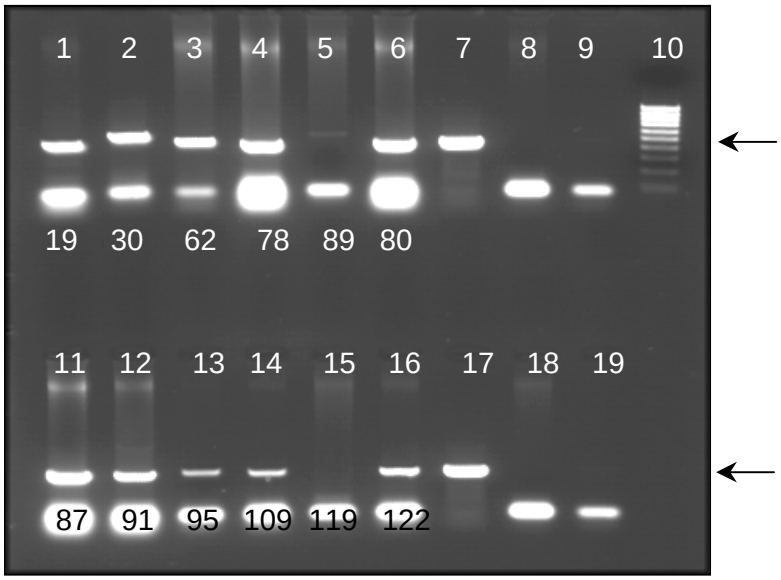


Figure 3 PCR products of ORF1/2 transgenic plants. Genomic DNA was extracted from transgenic ORF1/2 plants and amplified by PCR as described in Materials and Methods. Transgenic plants (numbers indicated at the bottom) are shown in lanes 1 to 6 and 11 to 16., lanes 7 and 17 is the positive control (plasmid DNA). Lanes 8, 18 and 9, 19 are the negative controls, untransformed *N. benthamiana* and water control, respectively. Lane 10 is the molecular size marker used. The arrow shows the expected 500 bp PCR product.

In the example shown in Fig. 4, in all cases tested it was possible to amplify the transgene by PCR. As shown in lane 16, no product could be obtained when genomic DNA from a vector-transformed line was used. The same is true for the negative controls included in this assay (DNA from untransformed *N. benthamiana* and water control, lanes 18 and 19, respectively).

To confirm that these PCR products corresponded to the amplification of the transgene, they were analysed by Southern blot. For this purpose they were loaded on an 1.2% agarose gel without ethidium bromide, run at 4 V/cm and transferred to a nylon membrane. The gel was not treated as normally done for Southern blot due to the small size of the PCR products. The membrane was incubated with a specific DIG labeled probe for each transgene. After detection with a chemiluminescent kit, positive signals could be observed, which corresponded to the PCR product of the transgenic plants. PCR of plasmids (pSV 5'3'S and pSV 5'3'AS) and of genomic DNA from non transformed *Nicotiana benthamiana* plants were used as positive and negative controls, respectively (data not shown). A water control was included in each case.

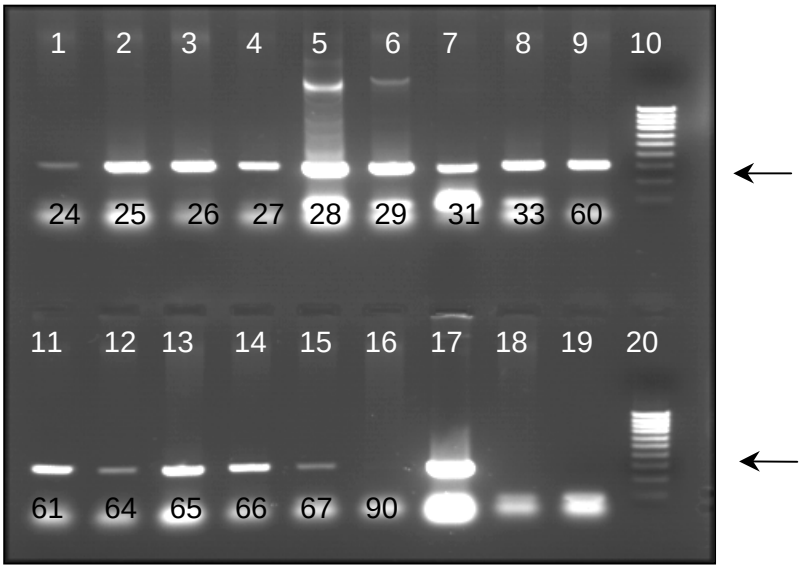


Figure 4 PCR products from 5‘3‘S and 5‘3‘AS plants. Genomic DNA was extracted from transgenic 5‘3‘S or 5‘3‘AS plants and amplified by PCR as described in Materials and Methods. Lanes 1 to 9 and 11 to 15 are transgenic plants (indicated at the bottom), lane 16 is a vector transformed plant, lane 17 is the positive control (DNA from plasmid) and lanes 18 and 19 are the negative controls, untransformed *N. benthamiana* and water control, respectively. The molecular size marker is shown on lanes 10 and 20 (100 bp marker).

3.1.4.2.3. PCR of nptII

When the amplification of genomic DNA from the transformed plantlets using the nptII primers was carried out, the expected PCR fragment could be detected in most cases (data not shown), indicating that the selection by kanamycin at this concentration (100 mg/l) was very effective. In this assay the genomic DNA purified from the transgenic vector-plants, which were transformed with the binary plasmid (pBin19), was tested for the insertion of the nptII gene.

In a few cases, even though the nptII sequence could be amplified by PCR it was not possible to detect the viral insert by the same method, despite the same genomic DNA was analysed in both cases. It is possible that during the transformation process the viral fragment was not transferred or not integrated into the genomic DNA of *N. benthamiana*.

3.1.4.3. Results from PCR and NPTII ELISA

When comparing the results obtained by these two methods it could be determined that 110 different transgenic lines of *N. benthamiana* were produced. These represent ca. 90% of the total plantlets that were able to regenerate and grow on selection medium.

Of the total, 50 plantlets contained the ORF1/2 sequence while 20 and 24 lines with the 5'3'S and 5'3'AS construct were generated, respectively. Sixteen plants contained the T-DNA region of the vector, i.e. plants which were only transformed with the binary vector pBin19.

A summary of these data is presented in Table 5. Only those plants which were positive when tested by NPTII ELISA were given a number and transferred to the greenhouse. In a few cases plants, which had tested positive by NPTII ELISA were negative by PCR when amplifying the nptII gene. In this case a new leaf sample was taken and assayed by ELISA, where it could be seen that they were negative (i.e. SV 119 in Tables 4 and 5). It is interesting to notice that as assessed by NPTII ELISA this plant had a relatively low value (0.43 ngNPTII/mgprot), which was close to the lower limit choosen to define a plant as negative (0.3 ngNPTII/mgprot).

ORF1/2				5‘3‘AS		5‘3‘S		Kp	
N°	ngNPTII/mgprot	N°	ngNPTII/mgprot	N°	ngNPTI/mgprot	N°	ngNPTII/mgprot	N°	ngNPTII/mgprot
19	0.45	127	1.27	24	0.84	26	1.21	90	0.54
23	0.34	128	1.24	25	0.45	31	2.89	103	0.35
30	0.71	129	1.65	27	0.35	32	0.87	105	1.12
62	0.87	131	0.78	28	2.74	33	3.47	106	0.74
78	0.45	133	0.78	29	0.98	61	1.30	107	1.10
80	1.80	141	0.75	60	1.10	64	0.55	114	1.30
87	2.10	142	1.45	65	0.68	69	0.98	115	0.57
91	0.62	143	0.35	66	1.23	88	2.13	123	1.87
95	0.31	144	0.65	67	1.54	99	1.41	130	0.78
98	4.27	145	1.32	68	0.75	100	1.70	132	0.85
108	2.90	146	2.11	77	2.70	101	1.76	138	2.70
109	0.86	147	0.45	79	0.68	102	1.79	152	1.40
110	2.48	148	1.45	81	1.23	104	0.79	157	0.65
111	0.44	149	0.78	82	1.14	134	3.10	158	3.12
112	2.81	150	0.41	83	0.87	135	3.40	159	2.13
113	0.94	151	0.66	84	0.54	136	1.38	160	1.78
116	0.95	153	1.78	85	0.54	137	1.65	Nb	0.00
117	4.50	154	0.45	86	1.93	139	1.87		
118	1.12	155	0.74	92	0.76	140	0.78		
119	0.43	156	1.02	93	3.13	165	1.65		
120	1.23	161	1.78	94	0.99				
121	0.77	162	0.97	96	1.32				
122	0.87	163	1.24	97	0.58				
124	0.64	164	0.54	125	3.42				

Table 3 NPTII ELISA values from primary *N. benthamiana* transformants. Values ranged from 0.4 to ca. 4,3 ngNPTII/mg prot. Before performing NPTII ELISA, leaf extracts of the plants tested were adjusted to 400 mg/ml of protein. Plants which showed OD_{405} readings less than 0.3 ngNPTII/mg prot were considered negative. Kp corresponds to plants transformed with the binary plasmid pBin19 alone. Nb is untransformed *N. benthamiana*.

3.1.5. Molecular characterisation of the transgenic lines

The detection of RNA transcripts expressed from transgenes is often an important step in the analysis of transgenic plants. These analyses may confirm that the RNA transcripts are of the expected size and allow its quantification in different transgenic lines. In most cases this is carried out by performing a Northern blot with help of a DIG labeled cDNA probe.

Five transgenic lines of each containing either the 5'3'AS or 5'3'S fragment, which would be further assayed in the greenhouse resistance tests were analysed by Northern blot (section 3.4). The expression of the transgene mRNA of some ORF1/2 plants is shown in Fig. 5. The expression of the nptII gene was also tested.

Ca. 10 to 20 µg of total RNA extracted from leaves of transgenic plants, from ca. 6 to 8 weeks old, was loaded on a formaldehyde gel. The gel was run at 4 V/cm during 4 hours and then blotted to a nylon membrane as described in Materials and Methods. The membrane was hybridized with a specific DIG labeled probe (see Materials and Methods). Detection of the transgenic mRNA was done by using a chemiluminescent method.

3.1.5.1. Northern blot of ORF1/2 lines

The mRNA produced by the expression of the ORF1/2 transgenic sequence should have an approximate size of 3,300 bp. The DIG labeled probe used to detect the transgenic RNA was complementary to the 5' end of the viral fragment.

After transfer and detection of the total RNA with a chemiluminescent kit, a single band of the expected size was found, indicating that the transgene was being expressed. No band was detected in the negative control, i.e. total RNA extracted either from vector transformed or non transformed *N. benthamiana* plants. In some cases a smaller band was also observed, which could be degradation products of the transgenic mRNA. Variable levels of expression of the transgene as determined by the intensity of the band were detected. As seen in Fig 5A, lines SV 98, SV 110 and SV 112 were high expressors of the viral transgene. Lines SV 87, SV 108 and SV 117 were low expressors (as determined in the original blot, but due to scanning, it can not be clearly seen in Fig. 5A). The other lines tested showed no expression of the transgene (i.e. SV 116, SV 122, SV 80; SV 145). In lane 12 a high background is observed due to the viral RNA present in the sample.

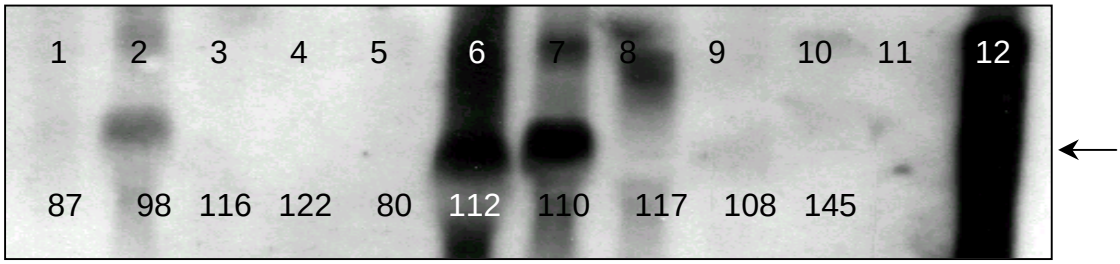


Figure 5A Northern blot from ORF1/2 transgenic lines. Total RNA extracted from transgenic lines was tested for the expression of the transgene using a ORF1/2 DIG labeled probe. Lanes 1 to 10 correspond to transgenic ORF1/2 lines (the lines are shown in the bottom), while lanes 11 and 12 represent healthy and BWYV infected untransformed *N. benthamiana* plants, respectively. The arrow shows the expected band of ca. 3,100 bp.

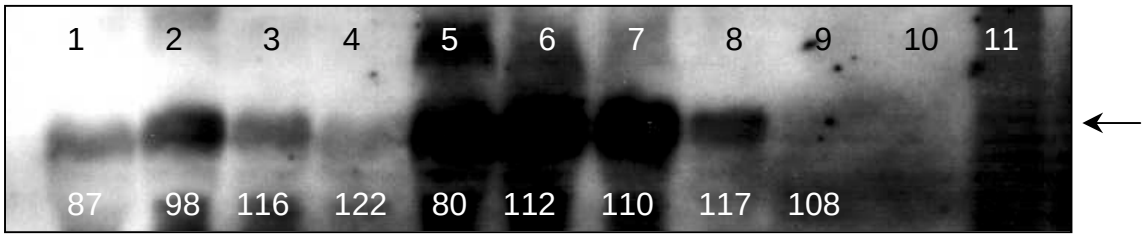


Figure 5B Northern blot of the nptII gene of transgenic ORF1/2 lines. Lanes 1 to 9 represent different lines (lines are shown in the bottom), while lanes 10 and 11 represent untransformed and BWYV infected *N. benthamiana* plants, respectively. The arrow indicates the position of the 1,200 bp band representing the mRNA of nptII.

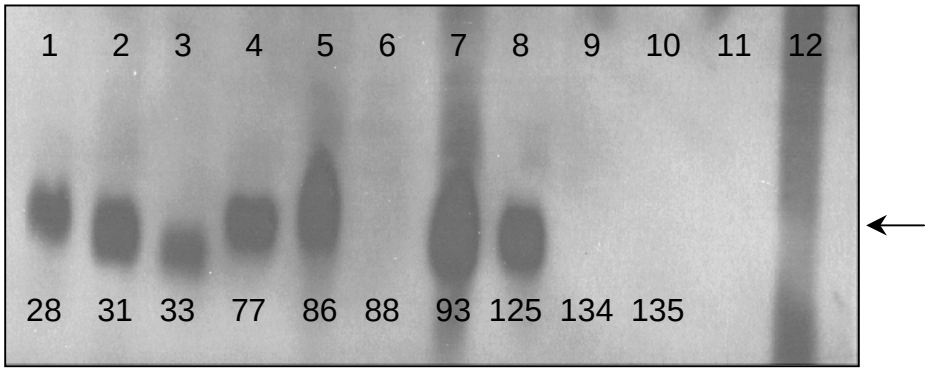


Figure 6 Northern blot from 5‘3‘S and 5‘3‘AS lines employed in the resistance tests. The expression of the 5‘3‘S or 5‘3‘AS transgene was assayed with a 5‘3‘S DIG labeled probe. Lanes 1 to 10 represent total RNA of the transgenic lines analysed (shown on the bottom). In lanes 11 and 12 total RNA from healthy and BWYV infected untransformed *N. benthamiana* are shown. The arrow shows the band of ca. 0.9 kb.

3.1.5.2. Northern blot of 5’3’S and 5’3’AS lines

Total RNA was extracted from young leaves of positive transgenic plants, containing either the 5’3’S or 5’3’AS insert. Since both constructs are quite similar, the RNA expression was detected using the same DIG-labeled probe.

As shown in Figure 6, a band of the expected size (app. 0.9 Kb) could be detected in most cases. Thus the transgene was expressed in these plants, although at different levels. RNA extracted from vector transformed or non transformed *N. benthamiana* plants did not react with the probe. The transgenic lines SV 28, SV 31, SV 33, SV 77, SV 125 expressed high levels of the transgene. Lines SV 86 and SV 93 showed very high levels of expression of the viral transgene, while in lines SV 88, SV 134 and SV 135 no expression was found as determined by Northern blotting. In lane 12 a high background is observed due to the viral RNA present in the sample.

No correlation could be found among lines containing either the sense or antisense construct and levels of expression of the transgene. Within the high expressors, lines SV 28, SV 77 and SV 125 carry the 5’3’AS construct, while SV 31 and SV 33 carry the 5’3’S construct. The two low expressing lines (SV 86 and SV 93) contain the 5’3’S construct and among the non expressing lines two (SV 134 and SV 135) contain the sense, while SV 88 carried the antisense sequence.

3.1.5.3. Northern blot of nptII

The transgenic plants generated in this study were transformed with the binary vector pBin19, which in the T-DNA region contains the nptII gene. The expression of the nptII mRNA was analysed. Total RNA was extracted and loaded on a gel as described in Materials and Methods (see 2.15.3.)

After prehybridizing and hybridizing with the nptII DIG labeled probe, a chemiluminescent method was used for detection. A single band, of ca. 1,300 bp which is of the expected size of the nptII mRNA was detected. As with the expression of the viral fragments, the level of RNA expression was variable in all lines studied (Fig. 5B). For three ORF1/2 lines high levels of expression could be observed (SV 80, SV 110 and SV 112), while other lines had low expression levels of the transgene, i.e. SV 108 and SV 117. The other lines tested had middle levels of expression of this transgene (Fig 6, lanes 1-4). It can be possible that due to high amounts of viral RNA present in the untransformed *N. benthamiana* plants assayed in parallel (Fig 5B, lane 11), a high background could be observed, however no band of the expected size of the nptII gene was detected. No correlation could be found between the level of RNA expression of the nptII gene and the amount of this protein determined by NPTII ELISA in these plants.

3.1.6. Seed viability and segregation of kanamycin resistance

The transgenic plants were transferred to soil and kept in the greenhouse, allowing them to flower and self-pollinate. They were normal in appearance and grew and developed similarly as non-transformed *N. benthamiana* plants.

Under the greenhouse conditions transgenic *N. benthamiana* plants required ca. 3 to 4 months to flower and self pollinate. Some of these lines failed to develop seeds (in total 18 lines) or produced very little amounts of them (4 lines). Seeds from the remaining lines (91 in total) were collected in order to assay for segregation of kanamycin resistance and resistance against BWYV inoculation in some transgenic lines (see section 3.2.).

Seeds (100-150 per line) collected from 91 different transgenic lines were surface sterilised with 70% ethanol and washed thoroughly with sterile water. They were placed on filter paper and kept for 7 to 10 days in a growth chamber under a “summer regime” (i.e. 16/8 h day/night at temperatures of 25°C/18°C, respectively). Four transgenic lines failed to germinate under these conditions. The germinated seeds of the remaining lines were transferred to MS medium containing 100 mg/l of kanamycin and allowed to grow for further 3 weeks in the growth chambers, under the same conditions indicated above.

Even though all T1 plants were positive for the presence of the nptII gene as tested by PCR or showed expression of the protein when performing NPTII ELISA, ca. one third of these transgenic lines had seeds which were either unable to germinate or when they germinated plantlets showed a very depressed growth, not developing more than their cotyledon stage.

The plantlets of the remaining transgenic lines developed well at 100 mg/l of kanamycin. However in some cases it was not easy to differentiate between plantlets that were Km^r or Km^s, therefore seeds were tested for germination at higher concentrations of this antibiotic (200 and 300 mg/l). Surface sterilised seeds (ca. 100 per line) were placed directly on MS plates containing different Km concentrations. As negative control, seeds (ca. 20) from non-transformed *N. benthamiana* were included on each MS plate. Plates were placed in the growth chamber and after ca. 3 to 4 weeks kanamycin resistant plantlets of the transgenic lines were able to grow even at 300 mg/l of antibiotic. At this same Km level seeds of non-transformed *N. benthamiana* germinated, but did not develop more than their cotyledon stage. At both Km concentrations tested resistant and sensitive plantlets could be easily detected. However there were some lines which had a very poor growth using any of the antibiotic concentrations, for example lines SV 146, SV 85 and SV 102, which showed a ca. 1:1 ratio of Km^r:Km^s plantlets. Only those transgenic lines that were able to grow at least at 100 mg/l of Km were further analysed in this study. A concentration of 200 mg/l was choosen to be further used to select npt^+ plantlets for the greenhouse resistance tests.

The χ^2 data are summarized in Table 4 for all lines tested, which were able to grow at 200 mg/l of Km. The data from lines which did not germinate or did not develop more than their cotyledon stage is not shown. For most lines a 3:1 segregation ratio of Km^r/Km^s seedlings was observed, indicating that the nptII gene was expressing from either a single or tightly linked loci. In a few cases 100% of resistant plantlets were observed, at the different Km concentrations tested. A χ^2 value of 3,84 is significant at the 0,05 probability level. From these data it can be seen that some lines had 2 copies of the transgene (i.e. SV 24, SV 132, SV 163, SV 33). When calculating the χ^2 value for these lines assuming two copies they are significant, i.e. 0,033 and 0,016 for lines SV 24 and SV 33, respectively.

ORF1/2			5‘3‘AS			5‘3‘S			Kp		
N°	Km^r:Km^s	χ^2	N°	Km^r:Km^s	χ^2	N°	Km^r:Km^s	χ^2	N°	Km^r:Km^s	χ^2
80	96:28	0.26	27	102:23	2.9	33	117:8	23.1	114	103:21	3.84
87	89:36	0.96	28	95:32	0	61	100:25	1.6	115	100:25	1.6
91	91:35	0.51	60	118:9	21.7	88	99:26	1.17	123	104:20	4.69
98	120:0		77	96:27	0.61	100	102:23	2.9	130	103:23	3.27
108	122:0		82	120:0		101	86:39	2.56	132	120:9	22.3
109	110:15	11.2	85	68:60	30.2	102	65:60	35.3	138	99:24	1.97
110	99:24	1.97	86	120:0		104	122:0		152	105:18	7.05
111	104:20	4.69	92	99:22	3	134	95:30	0.06	158	95:30	0.06
112	90:35	0.6	93	95:31	0.01	135	96:31	0.02	159	104:19	5.98
113	100:24	1.79	97	81:44	5.6	136	104:19	5.4			
116	85:42	4.4	125	96:29	0.22	137	102:23	2.9			
117	120:0					139	119:0				
118	85:38	2.28				140	103:21	3.8			
121	95:31	0.01				165	89:34	0.46			
122	96:29	0.22									
124	99:26	1.17									
127	95:31	0.01									
128	122:0										
129	100:25	1.6									
131	121:0										
141	95:32	0.0									
142	95:29	0.09									
145	86:42	3.1									
146	66:58	32.2									
148	100:25	1.6									
149	120:0										
150	76:48	13									
153	119:0										
156	105:21	4.67									
161	121:6	27.8									
162	91:34	0.32									
163	121:9	22.6									
164	121:0										
171	96:29	0.01									

Table 4. Results of $\chi 2$ obtained from plants growing on kanamycin. Km^r represents resistent plantlets, which could grow and develop at a concentration of 200 mg/l , while Km^s are the sensitive plants, which were not able to develop more than their cotyledon stage. Ratios were calculated from ca. 120 seeds per line. Untransformed *N. benthamiana* did not develop more than their cotyledon stage at this antibiotic concentration. In a few cases 100% germination was obtained at the different Km concentrations tested.

In a few cases where 100% of resistant plantlets were observed, it is possible that they integrated more than 3 copies of the transgene. This can be assumed, since in all cases the untransformed *N. benthamiana* seeds included as negative control in each plate were unable to develop more than their cotyledon stage. Because the viral sequence is adjacent to the marker gene in the T-DNA region transferred from the binary vector pBin19, it is assumed that the nptII gene and the viral transgene cosegregate in the progeny.

In Table 5 the results of PCR, NPTII ELISA and growth on Km for all transgenic lines assayed are summarized. Numbers were given only for those plants which had tested positive by NPTII ELISA. The results from PCR represent the amplification of either the viral transgene or the nptII gene. The results of Km+ indicates that seeds were able to germinate and plantlets developed more than their cotyledon stage, while the negatives, either seeds did not germinate or plantlets did not develop well at 100 mg/l of this antibiotic.

Some lines were analysed by Northern blot for the expression of the viral sequences or nptII gene. However it was not always possible to detect expression of the transgene. In some cases NPTII positive lines were tested for expression of the nptII gene, but no signal could be detected. In part this can be due either to a degradation of RNA or loss during blotting or problems with the DIG labeled probe(s) used for detection. Therefore only those lines selected to be tested for resistance against BWYV were analysed by Northern blot.

In summary due to the large number of transgenic plants obtained, only a few lines were further tested in resistance assays against BWYV. To select these lines, several criteria were taken into account, among them phenotype of the primary transformants, growth on Km (200 mg/l), levels of NPTII expression, number and quality of seeds produced.

In all cases the primary transformants were similar to untransformed *N. benthamiana*, developing a normal growth and phenotype. As described in section 3.1.6. not all plants were able to produce seeds or generated low levels of them and in some cases the seeds did not germinate at 100 mg/l of Km. At the same time, since most lines were able to grow at 200 mg/l of Km, the results of the χ^2 test was also considered as a parameter to decide which lines could be tested for resistance.

Line	Const.	PCR	N.blot	NPTII ELISA	Km
19	ORF1/2	+		+	+
23	ORF1/2	+		+	-
24	5'3'AS	+		+	+
25	5'3'AS	+		+	+
26	5'3'S	+		+	+
27	5'3'AS	+		+	+
28	5'3'AS	+	+	+	+
29	5'3'AS	+		+	-
30	ORF1/2	+		+	+
31	5'3'S	+	+	+	+
32	5'3'S	+		+	-
33	5'3'S	+	+	+	+
60	5'3'AS	+		+	+
61	5'3'S	+		+	+
62	ORF1/2	+		+	-
63	5'3'AS	-		-	-
64	5'3'S	+		+	-
65	5'3'AS	+		+	-
66	5'3'AS	+		+	-
67	5'3'AS	+		+	-
68	5'3'AS	+		+	-
69	5'3'S	+		+	-
77	5'3'AS	+	+	+	+
78	ORF1/2	+		+	-
79	5'3'AS	+		+	-
80	ORF1/2	+	+	+	+
81	5'3'AS	+		+	-
82	5'3'AS	+		+	+
83	5'3'AS	+		+	-
84	5'3'AS	+		+	-
85	5'3'AS	+		+	+
86	5'3'AS	+	+	+	+
87	ORF1/2	+	+	+	-
88	5'3'S	+	-	+	+

Line	Const.	PCR	N.blot	NPTII ELISA	Km
89	5'3'S	-		-	-
90	Kp	+		+	+
91	ORF1/2	+		+	+
92	5'3'AS	+		+	-
93	5'3'AS	+	+	+	+
94	5'3'AS	+		+	-
95	ORF1/2	+		+	-
96	5'3'AS	+		+	-
97	5'3'AS	+		+	+
98	ORF1/2	+	+	+	+
99	5'3'S	+		+	-
100	5'3'S	+		+	+
101	5'3'S	+		+	+
102	5'3'S	+		+	+
103	Kp	+		+	+
104	5'3'S	+		+	+
105	Kp	+		+	-
106	Kp	+		+	-
107	Kp	+		+	-
108	ORF1/2	+	+	+	+
109	ORF1/2	+		+	+
110	ORF1/2	+	+	+	+
111	ORF1/2	+		+	-
112	ORF1/2	+	+	+	+
113	ORF1/2	+		+	+
114	Kp	+		+	+
115	Kp	+		+	+
116	ORF1/2	+	+	+	+
117	ORF1/2	+	+	+	+
118	ORF1/2	+		+	+
119	ORF1/2	+		+	-
120	ORF1/2	+		+	-
121	ORF1/2	+		+	+
122	ORF1/2	+	+	+	+

Table 5. Summary of the characterisation of all transgenic lines generated in this study. See next page for explanation.

Line	Const.	PCR	N.blot	NPTII ELISA	Km
123	Kp	+		+	+
124	ORF1/2	+		+	+
125	5'3'AS	+		+	+
126	ORF1/2	+		+	-
127	ORF1/2	+		+	+
128	ORF1/2	+		+	+
129	ORF1/2	+		+	+
130	Kp	+		+	+
131	ORF1/2	+		+	+
132	Kp	+		+	+
133	ORF1/2	+		+	-
134	5'3'S	+	-	+	+
135	5'3'S	+	-	+	+
136	5'3'S	+		+	+
137	5'3'S	+		+	+
138	Kp	+		+	+
139	5'3'S	+		+	+
140	5'3'S	+		+	+
141	ORF1/2	+		+	+
142	ORF1/2	+		+	+
143	ORF1/2	+		+	-
144	ORF1/2	+		+	-
145	ORF1/2	+	+	+	+
146	ORF1/2	+		+	+
147	ORF1/2	+		+	-
148	ORF1/2	+		+	+
149	ORF1/2	+		+	+
150	ORF1/2	+		+	+
151	ORF1/2	+		+	-
152	Kp	+		+	+
153	ORF1/2	+		+	+
154	ORF1/2	+		+	-
155	ORF1/2	+		+	-
156	ORF1/2	+		+	+
157	Kp	+		+	-
158	Kp	+		+	+
159	Kp	+		+	+
160	Kp	+		+	-
161	ORF1/2	+		+	+
162	ORF1/2	+		+	+
163	ORF1/2	+		+	+
164	ORF1/2	+		+	+
165	5'3'S	+		+	+
171	ORF1/2	+		+	+

Table 5. (cont.) Summary of the characterisation of all transgenic lines generated in this study. PCR + means that the viral transgene could be amplified. Plants which were further tested in the greenhouse for resistance against BWYV were analysed by Northern blot. All lines were assessed by NPTII ELISA before transferring them to the glasshouse. Km is the ability of seeds to grow on Km selection media. Construct Kp represents the vector transfomed plants.

3.2. Greenhouse resistance tests

Two greenhouse resistance tests were performed to assay for BWYV resistance in some of the transgenic *N. benthamiana* lines produced. Plants were inoculated with the virus by means of the green peach aphid (*Myzus persicae*).

3.2.1. Selection of transgenic lines to be assayed in greenhouse resistance tests

Since a large number of transgenic lines were generated (ca. 70) it was decided to test a small number of lines per each construct. The choice of the transgenic lines to be assayed in the greenhouse resistance tests was based on a combination of criteria:

i) the data of the NPTII ELISA of the T1 lines. Plants which expressed high amounts of this protein were selected.
ii) the ability of seeds to develop and grow at different Km concentrations. Lines from which seeds were able to grow well and develop at 200 mg/l of Km.
iii) the results of the segregation tests. Plants that had χ^2 values which were not significant for a 3:1 ratio were eliminated. A few lines that had more than one copy of the transgene were tested.
iv) the amount of seeds produced per plant. In a few cases plants produced little amounts of seeds which would not be enough to be used in the greenhouse resistance tests (e.g. SV 60, SV 80 and SV 101).

The following transgenic *N. benthamiana* lines were tested for resistance against BWYV under greenhouse conditions:
a) 5’3’AS lines: SV 28, SV 77, SV 86, SV 93 and SV 125
b) 5’3’S lines: SV 31, SV 33, SV 88, SV 134 and SV 135
In this assay line SV 138 was choosen as vector-transformed control.

For the ORF1/2 transgenic lines the following lines were tested:
c) SV 98, SV 108, SV 110, SV 112, and SV 117. As vector transformed control line SV 158 was included.

The first greenhouse resistance test was carried out from June to August 1999 and the second from August to October 1999. Each resistance test was separated in two periods with a week interval, in order to facilitate the sampling for ELISA. Transgenic 5’3’AS or 5’3’S lines or the ORF1/2 lines were tested during the first and second period, respectively. In each assay a vector transformed line, as well as untransformed *N. benthamiana* plants were included as controls.

3.2.2. Preparation of transgenic plants for greenhouse resistance tests

Seeds (100-120) from the transgenic lines to be tested in the greenhouse were surface sterilised and allowed to germinate on filter paper for 1 week and then transferred to MS medium containing 200 mg/l of kanamycin. Germinated seeds of non-transformed *N. benthamiana* were placed on MS medium without antibiotic. In all cases plantlets grew well, showing a 3:1 segregation, although in a few cases 100% Km resistance was observed (lines SV 86, SV 98, and SV 108). In order to prove the efficiency of kanamycin selection plantlets of non-transformed *N. benthamiana* were included in each MS Km plate. After 2 to 3 weeks on selection medium, the non-transformed *N. benthamiana* plantlets were unable to develop more than their cotyledon stage.

Transgenic plants developed their first leaves after ca. 3 to 4 weeks on MS medium containing Km. They were transferred to pots and kept in the greenhouse. 60-80 plants from each line were distributed in two sets. One set was inoculated with BWYV and the other used as uninoculated control. Plantlets were well watered and protected with a plastic cover for at least 5 days. After this period of time, plastic covers were removed and 20 to 30 of the most healthiest plantlets were kept per set.

Random leaf samples from T2 seedlings of each line tested were assayed by NPTII ELISA for the product of the nptII gene. The presence of the integrated viral DNA insert in these T2 plants was positively verified by PCR analysis of genomic DNA, excluding any loss of the transgene in the segregation population of T2 seedlings (data not shown). Plants grew normally, showing no differences compared to the non-transformed control.

3.2.3. Inoculation of *N. benthamiana* plants with BWYV

Since BWYV can not be mechanically transmitted and it is limited to the phloem, the only possibility to inoculate the transgenic plants was with the use of one of the natural occurring vectors of this virus. BWYV is a persistently transmitted virus, therefore it is acquired by the vector in long AAPs ranging from 30 minutes up to days. After this period the vector can not transmit the virus immediately, since it has to circulate within the body of the insect to finally reach the salivary system. The commonly occurring aphid *Myzus persicae* was choosen as vector for transmission of BWYV in the greenhouse resistance tests.

Young fully expanded leaves of BWYV-infected oilseed rape (*Brassica napus*) were used as a source of virus for aphids. Non viruliferous *Myzus persicae* nymphs were allowed a 48 AAP on the leaves, before transfer to the different transgenic and non-

transformed *N. benthamiana* plants. No starving period is required, since it does not increase transmission of a circulative virus.

Five to seven viruliferous green peach aphids (*Myzus persicae*) were carefully transferred on newly developing leaves of each plant and allowed to feed for 3 to 4 days. Plants were protected with plastic covers to avoid the escape of aphids to the uninoculated controls. Even though theoretically one aphid per plant should be enough for virus transmission, more aphids are needed because usually not all aphids feed in a certain period and some drop from the plant on the soil. Aphids were eliminated using an insecticide, which was applied to infected and uninoculated plants. No apparent effect could be observed due to this compound. Aphids could not be detected on plants during the following 8 weeks of the experiment.

N. benthamiana plants were daily observed and ca. 15 days after infection, typical viral symptoms became visible, especially the yellowing of leaves in the infected non transgenic plants. At 4 wpi leaf discs were collected for further analysis by BWYV ELISA. At this same time, the height of all virus infected and not infected plants was measured. The same procedure was repeated at 6 and 8 wpi, at this last sampling time weight (excluding roots) of each infected and uninoculated plant was measured.

3.2.4. BWYV ELISA

Since its adaptation for plant viruses in 1977 by Clark and Adams, DAS ELISA has been widely used for virus detection, due to its easiness and relative cheap price compared to other methods.

To evaluate resistance against BWYV it is necessary to determine the presence of virus in the infected transgenic plants. Using DAS ELISA the virus can be detected as early as 3 wpi. In this study a minimal time of 4 wpi was allowed before performing this assay and two further sampling times were repeated at 6 and 8 wpi.

Leaf discs from three different levels (top, middle and bottom) from every viral infected plant were collected and immediately ground in sample buffer (PBS, Tween). In a few cases plants had a small number of leaves, therefore only a single leaf disk was collected. Samples were incubated overnight at 4°C with the primary antibody and detection was performed on the following day. The OD was read at 405 nm, OD_{405} values above 0.1 were considered as positive. Each plate included the respective negative controls (leaves from non-transformed uninoculated *N. benthamiana* and samples from non-infected transgenic plants). No differences in the OD_{405} readings between the negative control, the non-infected transgenic lines and the blank could be found.

3.2.5. First Greenhouse Resistance Test

The first greenhouse resistance test was carried out from May 31st to August 24th 1999. Plants were kept in the greenhouse with a daily temperature of 23°C for 18 hours and 16°C for 6 hours at night. Under these conditions transgenic and non-transformed *N. benthamiana* plants showed normal growth and development during the 8 weeks of the experiment. No symptoms due to other diseases or nutritional deficiency in the *N. benthamiana* plants could be observed.

The average OD_{405} readings of the different transformed lines tested for each BWYV ELISA are summarized in Table 6. The individual data for each line can be found in the Appendix.

In general all the 5'3'AS transgenic lines analysed showed mainly no differences in their response to virus inoculation, when compared to the BWYV infected vector-control and non-transformed group. On the other hand, some of the 5'3'S lines tested had lower OD_{405} values than the virus inoculated controls at the end of the experiment. In the case of *N. benthamiana* plants transformed with the viral replicase of BWYV (ORF1/2), three out of the 5 lines studied had lower OD_{405} readings than the virus infected controls. The BWYV challenged controls (vector- and non-transformed *N. benthamiana* plants) responded similarly, developing typical symptoms due to BWYV, therefore being completely susceptible to the virus.

The average results of ELISA at 4, 6 and 8 wpi are shown for each experiment (Table 6), as well as the final height (Fig. 7, 8 and 9) and weight (Table 7) of infected and healthy plants. The individual data of height at 4 and 6 wpi and those of ELISA and final weight are included in the Appendix.

3.2.5.1. Response of control plants to BWYV inoculation

To verify if the green peach aphids transmitted successfully the virus into the plants, non-transformed *N. benthamiana* plants were included as a control in the resistance tests. The rate of infection of these plants should be 100%, since they are completely susceptible to BWYV.

At the same time vector transformed *N. benthamiana* plants must be included in each test to exclude that any possible resistance observed in the transgenic lines is due to the transferred region of the binary vector or to any somaclonal variation in the plant genome, but to the viral sequence itself. These plants should also be susceptible to BWYV. In this study two vector lines, SV 138 and SV 158 were tested.

According to the results of ELISA at 4 wpi for each experiment it was found that 100% and only in one case 95% (untransformed *N. benthamiana* in the second greenhouse resistance test) of the total virus challenged plants of the controls were infected by BWYV. These results were confirmed when performing this assay with leaf samples taken at 6 and 8 wpi.

The uninoculated plants of the two vector transformed plants included as controls developed normally, reaching an average final height of 60 cm and weight of ca. 18 g during the first resistance test. The uninoculated untransformed *N. benthamiana* plants reached an average final height and weight of 58 cm and 18 g, respectively.

There was no difference in the development between the two different vector control lines tested (SV 138 and SV 158). In both cases the virus inoculated plants developed typical BWYV symptoms such as stunting and yellowing of their leaves. The final height of the BWYV infected non-transformed and vector transformed plants was strongly reduced reaching only ca. 30% to 50% of the final height of the respective healthy plants. This reduction in growth could be detected as early as 4 wpi and was kept constant during the experiment.

The final weight of the virus infected vector transformed and untransformed *N. benthamiana* plants, was drastically reduced. In some cases it reached only 20% of the weight found in the uninoculated plants, while in one case it was about 45% of the average from the healthy plants (untransformed plants in the second greenhouse resistance test).

During the first resistance test some virus inoculated plants of these lines died before completing 8 weeks in the greenhouse, due to the high degree of infection by the virus. Therefore it was not possible to assay them by ELISA, but the final height and weight was measured.

First Resistance test

A

Line	ELISA 4wpi	ELISA 6wpi	ELISA 8wpi
98	1.252 ± 0.77	0.893 ± 0.87	0.560 ± 0.63
108	0.728 ± 0.43	1.014 ± 0.43	0.639 ± 0.52
110	0.626 ± 0.35	0.830 ± 0.47	1.476 ± 0.62
112	0.401 ± 0.20	0.824 ± 0.51	1.716 ± 0.81
117	0.720 ± 0.45	0.246 ± 0.20	0.275 ± 0.34
158	0.943 ± 0.35	1.775 ± 0.57	2.525 ± 0.56
Nb	1.676 ± 0.59	1.621 ± 0.40	2.098 ± 0.45

B

Line	ELISA 4wpi	ELISA 4wpi	ELISA 4wpi
31	0.403 ± 0.28	1.534 ± 0.58	2.301 ± 0.83
33	0.218 ± 0.19	0.448 ± 0.31	0.371 ± 0.43
88	0.203 ± 0.23	0.854 ± 0.38	1.820 ± 0.78
134	0.369 ± 0.32	1.357 ± 0.81	1.164 ± 0.51
135	0.293 ± 0.22	1.466 ± 0.58	1.873 ± 0.94

C

Line	ELISA 4wpi	ELISA 6wpi	ELISA 8wpi
28	0.455 ± 0.34	1.503 ± 0.74	2.921 ± 1.00
77	0.240 ± 0.27	0.615 ± 0.39	0.600 ± 0.43
86	0.283 ± 0.24	1.550 ± 0.64	2.297 ± 1.24
93	0.247 ± 0.16	0.635 ± 0.48	2.220 ± 0.96
125	0.508 ± 0.43	1.160 ± 0.38	2.464 ± 0.98
138	0.504 ± 0.37	1.927 ± 0.60	2.656 ± 0.70
Nb	0.545 ± 0.34	1.524 ± 0.62	2.469 ± 0.86

Second Resistance test

A

Line	ELISA 4wpi	ELISA 6wpi	ELISA 8wpi
98	0.706 ± 0.55	1.285 ± 1.22	1.237 ± 1.35
108	0.678 ± 0.72	1.052 ± 0.97	1.144 ± 1.18
110	0.484 ± 0.50	0.937 ± 0.85	1.050 ± 0.74
112	0.270 ± 0.33	0.789 ± 0.71	0.998 ± 0.88
117	1.104 ± 0.66	1.694 ± 0.86	1.672 ± 0.73
158	1.154 ± 0.61	1.864 ± 0.28	2.176 ± 0.59
Nb	1.108 ± 0.62	2.143 ± 0.87	2.200 ± 0.86

B

Line	ELISA 4wpi	ELISA 4wpi	ELISA 4wpi
31	0.878 ± 0.61	1.003 ± 0.64	1.304 ± 0.89
33	1.092 ± 0.66	1.118 ± 0.55	0.554 ± 0.53
88	1.054 ± 0.48	1.279 ± 0.54	1.591 ± 0.62
134	0.849 ± 0.68	0.953 ± 0.71	0.622 ± 0.42
135	1.042 ± 0.26	1.348 ± 0.31	1.577 ± 0.67

C

Line	ELISA 4wpi	ELISA 6wpi	ELISA 8wpi
28	0.569 ± 0.41	0.967 ± 0.65	1.283 ± 0.99
77	0.393 ± 0.40	0.618 ± 0.36	0.618 ± 0.40
86	0.921 ± 0.46	1.495 ± 0.69	1.637 ± 0.78
93	1.149 ± 0.68	1.398 ± 0.90	1.780 ± 0.92
125	1.249 ± 0.69	1.783 ± 0.69	2.048 ± 0.93
138	1.291 ± 0.49	1.483 ± 0.59	1.724 ± 0.51
Nb	1.322 ± 0.36	1.709 ± 0.43	1.992 ± 0.56

Table 6 Summary of the BWYV ELISA values of both greenhouse resistance tests. For each transgenic line, 20 plants were inoculated with the virus via green peach aphids (5 to 7 aphids per plant). The results represent the average OD_{405} readings of each line at 4, 6 and 8 weeks post infection (wpi). Transgenic ORF1/2, 5‘3‘S and 5‘3‘AS lines are shown in panels A, B and C, respectively. The corresponding vector controls (SV 158 and SV138 for ORF1/2 and for 5‘3‘S and 5‘3‘AS, respectively) and untransformed N. benthamiana (Nb) are shown in each case.

3.2.5.2. Response of ORF 1/2 lines to BWYV inoculation

Five ORF1/2 *N. benthamiana* transgenic lines were assayed in the greenhouse for resistance against BWYV; namely lines SV 98, SV 108, SV 110, SV 112 and SV 117. As controls a vector transformed line (SV 158) as well as non-transformed *N. benthamiana* plants were included. For each line 20 plantlets were inoculated with BWYV and other 20 plantlets were kept as healthy controls. No major differences in the growth of the lines assayed could be observed before transferring aphids to the plants. After inoculating the plants with BWYV they were analysed for virus at 4, 6 and 8 wpi by a BWYV ELISA. During the period of the experiment no other alterations, besides those due to viral infection could be observed.

Lines SV 117, SV 108 and SV 98 showed a stronger response to viral inoculation as determined by ELISA and height measured at 4, 6 and 8 wpi. On the other hand, lines SV 110 and SV 112 behaved similar to the infected controls developing typical viral symptoms due to BWYV.

3.2.5.2.1. BWYV ELISA

The results of the BWYV ELISA at 4 wpi showed that the mean OD_{405} readings of the infected plants of two lines, SV 110 and SV 112 were lower than the averages observed in the other virus inoculated lines tested. These values increased when assayed at 6 and 8 wpi, reaching similar levels to those of the virus infected controls (Table 6A).

The other three lines tested (SV 98, SV 108 and SV 117) had equal or slightly higher mean ELISA values at 4 wpi than both infected controls. Although the average OD_{405} increased at 6 wpi, it decreased when assayed at 8 wpi. In general lower levels of virus were found in these lines at 8 wpi than at 4 or 6 wpi, which were below the levels found in both BWYV infected controls (Table 6A). For lines SV 98 and SV 117, this represented a ca. 2.5 times reduction in their average OD_{405} compared to the values obtained at 4 wpi. The individual analysis of the data from the infected plants of these three transgenic lines showed that not all plants had decreased ELISA values at 8 wpi.

In order to exclude any error possibility during sampling, the ELISA was repeated. Leaf discs were taken from different levels (bottom, middle and upper stages) of the virus infected plants of lines SV 98, SV 108 and SV 117 and further processed as described in Materials and Methods. The result of this second ELISA was similar to the first one, i.e. the OD_{405} readings were lower than those found at 4 and 6 wpi for some plants of these lines. Contrarily, the OD_{405} values from the virus infected controls constantly increased when tested at 4, 6 and 8 wpi as determined by BWYV ELISA (Table 6A).

The results of DAS ELISA showed that in line SV 108, 12 out of the 20 (60%) virus infected plants tested had lower OD_{405} values than those detected at 4 and 6 wpi for the same plants. The same could be observed for lines SV 117 and SV 98 in which 17 and 14 of the total plants tested (representing 85% and 70% of the total virus infected plants, respectively) had lower OD_{405} at 8 wpi than at 4 or 6 wpi (Table 10). Interestingly, the rest of the virus inoculated plants of these lines behaved similar to the infected controls. These results will be shown with more detail (section 3.3.9.).

3.2.5.2.2. Height

The height of the BWYV challenged and uninoculated *N. benthamiana* plants was measured at 4, 6 and 8 wpi in order to have an additional parameter of the response of plants to viral inoculation.

Uninoculated plants of lines SV 98, SV 108 and SV 117 reached similar final heights as the controls (60 cm). The uninoculated plants of lines SV 110 and SV 112 reached the a lower final height (Figure 7).

The final height of virus infected plants of all lines tested was lower than their respective uninoculated controls. The most drastic effect could be observed in lines SV 110 and SV 112 and in both infected controls. In all these cases the infected plants reached an average final height ca. 30% to 40% of the respective healthy ones. The decreased growth of healthy as well as infected plants of lines SV 110 and SV 112 could be detected at 4 wpi and this ratio remained constant during the entire experiment.

The final average height of the infected plants from the other three lines analysed was higher than the one observed in the virus challenged controls. The BWYV infected plants of lines SV 117, SV 108 and SV 98 had reduced their growth by 27%, 30% and 33%, respectively compared to their healthy controls (Fig. 7). The virus infected plants of line SV 117 reached in average 67% of the height of the uninoculated at 4 wpi and this value increased at 8 wpi (74%). On the other hand, the height of virus inoculated plants of line SV 98 did not decrease drastically at 4 wpi, reaching in average 84% of the value of the respective healthy control plants, however the final height of these plants represented 67% of the uninoculated plants.

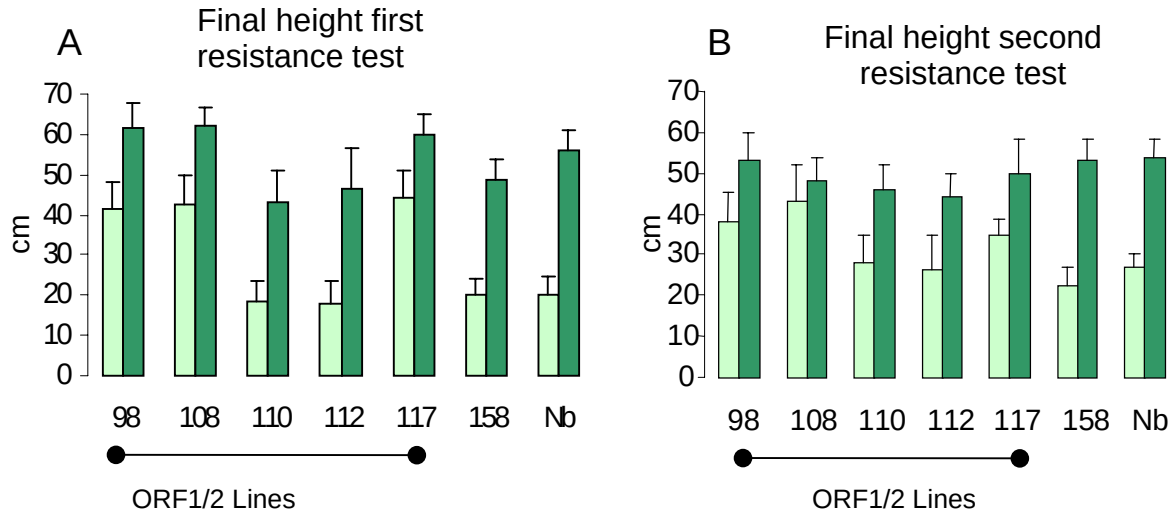


Figure 7 Final height values measured at 8 wpi in ORF 1/2 plants. In A and B, the average heights at the end of the experiment for the infected (▫) and healthy (▪) plants are shown. Each bar represents the average value of 20 infected or healthy plants (± SD). SV 158 is the vector transformed line, Nb is non transformed *N. benthamiana*.

3.2.5.2.3. Final weight

The final weight of viral infected and healthy plants was included as an indicator of the transgenic plants to BWYV (i.e. measured at 8 wpi).

No difference between the average final weight (ca. 21 g) of the uninoculated plants of the ORF1/2 lines tested with those of the controls was found (Table 7A).

On the one hand, the final weight of the BWYV inoculated plants of lines SV 110 and SV 112 was significantly decreased compared to the respective uninoculated controls (Table 7A). The weight ratio (infected vs healthy) was ca. 0.2 for each of these lines, which was similar to the one calculated for both controls.

On the other hand, the average final weight of the infected plants from the other lines tested was not drastically reduced. The best weight ratio (0.67) was determined for line SV 98, while BWYV infected plants of lines SV 108 and SV 117 reached ca. 56% of the final weight of the respective uninoculated control (Table 7A).

In general, these results clearly showed that none of these lines were resistant to BWYV. However, three of the five lines assayed, SV 98, SV 108 and SV 117 had a slightly better tolerance to BWYV, when the average ELISA values, final height and weight of the challenged plants of these lines are compared to those of the controls.

A

Line	First resistance test W. inf. 8wpi	First resistance test W. cont. 8wpi	Second resistance test W. inf. 8wpi	Second resistance test W. cont. 8wpi
98	14.9 ± 5.3	22.28 ± 3.7	6.2 ± 2.0	11.0 ± 4.3
108	13.4 ± 7.6	22.83 ± 3.5	9.5 ± 2.8	8.0 ± 3.1
110	3.6 ± 1.9	18.65 ± 6.0	5.3 ± 2.8	10.7 ± 4.3
112	3.7 ± 2.7	21.7 ± 6.6	6.7 ± 3.7	10.6 ± 3.7
117	11.7 ± 4.5	21.14 ± 4.1	7.0 ± 2.8	9.5 ± 4.8
158	4.1 ± 1.5	18.84 ± 5.6	3.4 ± 1.1	13.0 ± 5.1
Nb	3.9 ± 2.3	19.32 ± 6.3	5.0 ± 1.9	11.5 ± 3.

B

Line	W. inf. 8wpi	W. cont. 8wpi	W. inf. 8wpi	W. cont. 8wpi
31	5.4 ± 2.3	19.7 ± 6.2	8.2 ± 3.4	11.8 ± 4.1
33	14.2 ± 7.8	23.6 ± 9.2	8.8 ± 1.5	13.3 ± 3.2
88	3.1 ± 2.1	16.2 ± 7.7	5.1 ± 2.0	9.7 ± 3.2
134	6.7 ± 2.7	21.0 ± 6.2	8.1 ± 2.7	12.0 ± 4.5
135	5.7 ± 2.1	21.1 ± 9.9	4.6 ± 1.9	12.4 ± 4.5
138	4.0 ± 1.6	16.9 ± 7.4	4.4 ± 1.5	11.0 ± 5.4
Nb	3.5 ± 1.4	17.4 ± 7.2	4.5 ± 1.7	12.2 ± 4.3

C

Line	W. inf. 8wpi	W. cont. 8wpi	W. inf. 8wpi	W. cont. 8wpi
28	4.0 ± 1.9	21.8 ± 8.0	6.7 ± 3.1	12.6 ± 3.0
77	5.4 ± 2.2	22.0 ± 8.0	5.7 ± 1.8	10.2 ± 2.3
86	5.9 ± 2.1	20.7 ± 7.0	5.2 ± 1.9	11.8 ± 4.0
93	7.9 ± 3.2	18.5 ± 6.0	7.2 ± 2.2	12.0 ± 3.4
125	4.1 ± 1.9	16.6 ± 7.2	4.6 ± 1.9	9.0 ± 5.2
138	4.0 ± 1.6	16.9 ± 7.4	4.4 ± 1.5	11.0 ± 5.4
Nb	3.5 ± 1.4	17.4 ± 7.2	4.5 ± 1.7	12.2 ± 4.3

Table 7 Final weight (in g) of infected (W. inf.) and healthy plants (W. cont.) assayed for resistance in the greenhouse. The weight from each plant was measured at 8 wpi. Each value represents the average of 20 plants. In A, B and C the results for transgenic lines ORF1/2, 5‘3‘S and 5‘3‘AS are shown, respectively. Lines SV 138 and SV 158 correspond to the vector controls used. Nb is untransformed *N. benthamiana*.

3.2.5.3. Response of 5'3'S lines to BWYV inoculation

Five 5'3'S transgenic lines were assayed in greenhouse resistance tests. They were SV 31, SV 33, SV 88, SV 134 and SV 135. According to the response of the infected plants to BWYV it was possible to classify them in two groups. Three transgenic lines were completely susceptible to viral infection (SV 31, SV 88 and SV 135), while lines SV 33 and SV 134 showed milder viral symptoms.

3.2.5.3.1. BWYV ELISA

Compared to the infected controls low average BWYV ELISA values of the 5'3'S virus infected plants were found at 4 wpi (Table 6B). In leaf samples from some plants of these lines, no BWYV could be detected by this method (see e.g. ELISA results of SV 33 plants in the Appendix).

The most susceptible lines to BWYV as determined by ELISA were SV 88 and SV 135. Although these lines had relatively low virus titers at 4 wpi, these levels increased at 6 and 8 wpi being comparable to those found in BWYV infected control plants. However, the average OD_{405} from line SV 88 at 6 wpi was still slightly below the mean value found in both infected controls. Virus inoculated plants of line SV 31 had low initial ELISA values, but these increased drastically when measured at 8 wpi, reaching similar levels as those found in the infected controls (Table 6B).

Low average OD_{405} readings in BWYV inoculated plants of line SV 33 at 4 wpi were found. These values tended to increase slightly at 6 wpi representing ca. 25% of those determined for the infected controls. OD_{405} readings at 6 wpi and 8 wpi were similar in these plants, but in the latter it represented a ca. 6 times reduction compared to the infected controls. Although average OD_{405} readings at 6 wpi and 8 wpi of this line were similar, the analysis of individual data of the inoculated plants showed that values tended to decrease in most cases (see section 3.3.9.).

Although virus challenged plants of line SV 134 had ELISA values similar to those of the controls at 6 wpi, a ca. 2 times reduction in the viral levels in this line was found at the end of the experiment (Table 6B).

An interesting response was observed in the virus infected plants of line SV 33. The average values of ELISA at 4, 6 and 8 wpi were always below (at least 50%) those for the BWYV infected controls. The average OD_{405} reached a maximum at 6 wpi and decreased significantly when assayed at 8 wpi (representing ca. 80% reduction compared to the infected controls). In some cases the OD_{405} values were even lower than those observed at 4 or 6 wpi for the same plants (section 3.3.9.).

3.2.5.3.2. Height

Uninoculated plants of the 5’3’S lines tested had an average final height of 60 cm, similar to the one of the controls (59 cm). Line SV 88 had a decreased final height (80% of the other transgenic lines), while for line SV 33 the mean height (67 cm) was slightly above the group average (Fig 8).

BWYV infected plants of lines SV 31 and SV 88 had a reduced growth (ca. 50%), compared to their healthy controls at 4 wpi and this difference was kept constant until the end of the experiment. When measuring the height of the BWYV infected plants of line SV 135 it was found that they reached in average 65% and 50% of the growth of the healthy control at 4 and 8 wpi, respectively (Fig. 8).

A ca. 25% and. 40% decrease in the virus inoculated plants of lines SV 33 and SV 134 compared to the respective controls was found (Fig 8). The BWYV infected vector transformed and non-transformed *N. benthamiana* plants reached in average 50% of the height of the uninoculated controls at 4 wpi, keeping this ratio constant until the end of the greenhouse resistance test.

3.2.5.3.3. Final weight

Similar average final weights were measured in the uninoculated plants of the transgenic lines and controls analysed in this resistance test (22 g). The only exception was line SV 88 which had an average final weight of 16 g.

BWYV challenged plants of line SV 88 had an extremely low final weight (3 g) which represented only 20% of the respective healthy control (Table 7B). This result is in accordance with the low final height observed in this line compared to the other transgenic lines tested. Inoculated plants of line SV 31 had a reduced average final weight similar to the one found in the infected controls (ca. 30%).

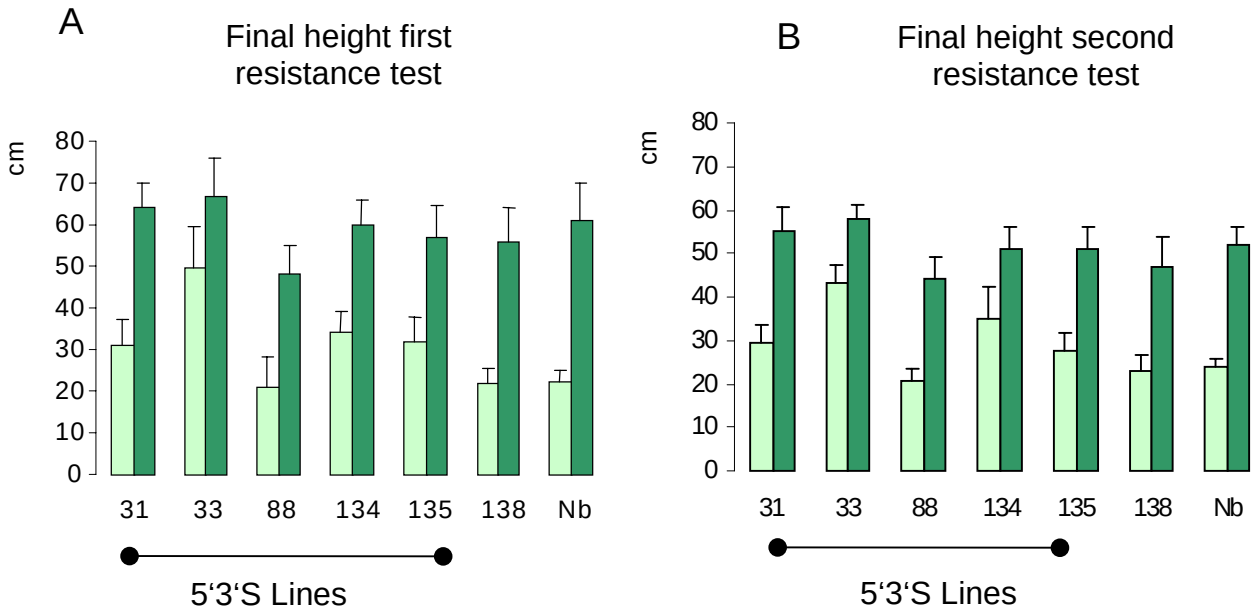


Figure 8 Final height values measured at 8 wpi for 5‘3‘S lines. In A and B, the average heights at the end of the experiment for the infected (□) and healthy (■) plants for the first and second greenhouse resistance test are shown, respectively. Each bar represents the average value of 20 infected or healthy plants. SV 138 corresponds to the vector transformed line employed, Nb is non transformed *N. benthamiana*.

The final weight ratios of the infected vs the uninoculated plants from three of the transgenic lines analysed were not as reduced. Values of 0.6 for line SV 33, 0.5 for line SV 134 and 0.4 for line SV 135 were determined, which are above those calculated for the untransformed and vector transformed plants (ca. 0.3).

The results of BWYV ELISA, final height and weight of the virus inoculated 5’3’S transgenic plants studied in this greenhouse resistance test showed that lines SV 33 and SV 134 had a slightly stronger response to BWYV compared to the inoculated plants of the controls. Even though line SV 88 had a weak protection against BWYV according to the OD_{405} readings at 8 wpi, the average final height and weight of the virus challenged plants were similar to those observed in both virus infected controls.

3.2.5.4. Response of 5'3'AS lines to BWYV inoculation

The five 5'3'AS transgenic lines tested for BWYV resistance under greenhouse conditions were SV 28, SV 77, SV 86, SV 93 and SV 125. Plantlets developed well in kanamycin (200 mg/l), having a 3:1 segregation ratio. They were transferred to pots in the greenhouse and after a week, aphids were transferred on new leaves and allowed to feed for 3 to 4 days before being eliminated with an insecticide. Healthy plants developed well, showing no phenotypical differences as compared to non-transformed plants. All virus infected transgenic *N. benthamiana* plants developed symptoms due to BWYV after ca. 2 to 3 wpi. After one month they were stunted and showed yellowing of their leaves. The study was carried out for 8 weeks after viral infection.

3.2.5.4.1. BWYV ELISA

The OD_{405} readings of the 5'3'AS lines tested at 4 wpi were similar to the average values obtained for both infected controls. Three lines (SV 77, SV 86 and SV 93) had slightly lower mean values (Table 6C).

Lines SV 77 and SV 93 had average OD_{405} readings at 6 wpi below those observed in the infected controls (Table 6C). The analysis of the BWYV ELISA data determined at 8 wpi showed that only line SV 77 kept relatively low average values, which represented ca. 25% of the average from the infected controls. No differences in the average final OD_{405} readings of the other four 5'3'AS lines and both infected controls tested were found. In all cases these values increased with time (Table 6C).

3.2.5.4.2. Height

In general, healthy plants of the five 5'3'AS transgenic lines and those of the controls reached similar average final heights (58 cm). Line SV 125 was only 50 cm, while line SV 86 developed to a final height of 64 cm.

Virus infected plants of four of the transgenic lines tested (SV 28, SV 77, SV 86 and SV 125) were only 50% as high as the respective controls at 4, 6 and 8 wpi. (data in Appendix and Fig. 9). This was also true for line SV 77, which had the lowest mean ELISA value (see 3.3.5.4.1.). The only exception was line SV 93, where the virus inoculated plants maintained ca. 60% of the final height of its healthy control. The final average height of the BWYV infected plants of both controls, SV 138 and non-transformed *N. benthamiana* was 50% of the respective healthy control (Fig. 9).

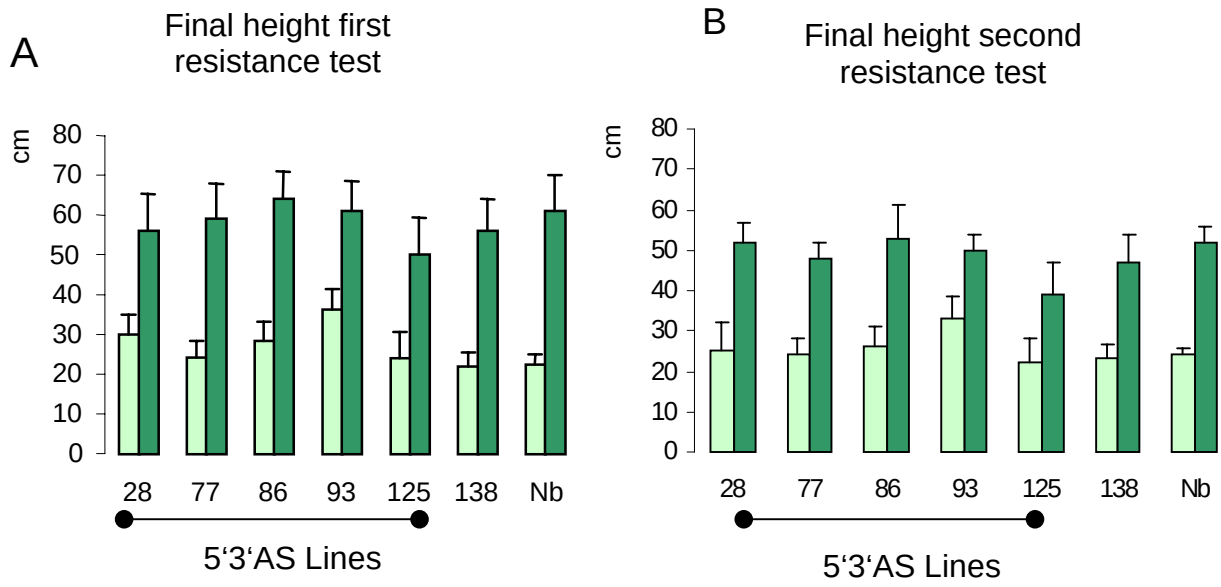


Figure 9 Final height values measured at 8 wpi of plants containing the 5‘3‘AS construct. In A and B the. final height of infected ( ) and healthy ( ) plants of each line is shown. Each bar represents the average value of 20 infected or healthy plants. SV 138 corresponds to the vector transformed line employed, while Nb represents the non transformed *N. benthamiana*

3.2.5.4.3. Final weight

The average final weight of the 5’3’AS uninoculated plants was ca. 20 g. This value was slightly lower in both controls (17 g), as well as in line SV 125, which also had a final height below the average of the other transgenic lines assayed in parallel.
The final weight of the BWYV challenged plants of the 5’3’AS lines tested was ca. 25% of that of the respective healthy plants. The weight ratio (ca. 40%) of line SV 93 was slightly above the average determined for the other lines. Both infected controls reached ca. 30% of the final weight of the healthy plants (Table 7C).

In summary, when all parameters of the five virus inoculated 5’3’AS transgenic lines were compared to those of the infected controls no differences in the response to BWYV could be found. Therefore these lines performed like controls, showing no resistance against BWYV. On the one hand, the infected plants of line SV 77 had significant low OD_{405} values during the entire experiment, but their final height and weight were similar to those of the infected controls. On the other hand, virus inoculated plants of line SV 93 had OD_{405} readings similar to those of the virus infected controls, although the final average height and weight of this line were slightly higher than those observed for the infected plants of the other lines tested.

3.2.6. Second greenhouse resistance test

The second greenhouse resistance test was performed from the 31st of July to the 30th of October 1999. In this case the same procedure described for the first greenhouse resistance test was followed. Artificial light was supplied in order to keep the 16/8 hours day/night conditions used in the first test. No visible alterations besides the typical viral infection symptoms could be observed in the *N. benthamiana* plants assayed during this period. In this experiment a lower viral infection rate of the BWYV challenged transgenic plants than in the first resistance test was determined. A summary of the data obtained can be seen in Tables 6 and 7. The individual data for each line can be found in the Appendix.

3.2.6.1. Response of ORF1/2 lines to BWYV inoculation

In this second greenhouse resistance test, 80% and 70% of the plants of line SV 110 and SV 112 were infected with BWYV as assessed by ELISA, respectively. The vector transformed controls showed 100% infection, while 95% of the total infected untransformed *N. benthamiana* plants were positive for BWYV as determined by ELISA. For the other transgenic lines tested in parallel it is assumed that 80% to 90% of the challenged plants were infected by the virus. In this case it is difficult to differentiate plants which were initially infected from those that had escaped infection. This is based mainly on the results observed in the first greenhouse test, where although some plants were infected by BWYV they had low ELISA values. Despite these results, most lines showed a similar response to virus inoculation as in the first greenhouse resistance test.

3.2.6.1.1. BWYV ELISA

All ORF1/2 lines assayed were susceptible to BWYV as determined by ELISA at 4, 6 and 8 wpi. No major differences between these results and those of the infected controls tested at the same sampling times were found (Table 6A). Lines SV 110 and SV 112 had relatively low average OD_{405} readings at all times analysed, which are similar to the results found in the first resistance test, although in the latter it was only observed at 4 and 6 wpi.

The average OD_{405} readings of infected plants of the lines tested increased at 6 wpi and remained constant when tested at 8 wpi, reaching similar levels to those of both BWYV infected controls. In this greenhouse resistance test a large variability was found in the data of BWYV ELISA (determined as SD), especially in lines SV 98 and SV 108. In these two lines inoculated plants could be classified in two groups according to the OD_{405} readings (see section 3.3.9.).

3.2.6.1.2. Height

Uninoculated plants from the transgenic lines tested reached an average final height of 49 cm, not differing from that of the controls (50 cm). As in the first greenhouse resistance test, the final height of lines SV 110 and SV 112 was slightly below the average of the group (ca. 90%, Fig. 7).

The average final height of the BWYV infected plants of lines SV 110 and SV 112 represented ca. 40% of the respective uninoculated controls. A similar reduction was also determined in the virus infected controls (Fig. 7).

BWYV inoculated plants of lines SV 98, SV 108 and SV 117 had an average final height which was not drastically decreased. The mean values represented 70% to 90% of those of the respective healthy plants. It is interesting to note that line SV 108 reached in average 90% of the final height of its healthy control, although it had a high ELISA average value (Fig. 7).

3.2.6.1.3. Final weight

Uninoculated plants of the ORF1/2 lines tested weighted in average 10 g, which was slightly lower than the one observed in both controls (ca. 12 g).

The virus inoculated plants of lines SV 110 and SV 112 as those of the controls had similar average final weights (ca. 4 g), which represented 30% of that determined in the respective uninoculated plants.

BWYV challenged plants of lines SV 98 and SV 117 reached in average 70% of the final weight of their respective uninoculated controls. For line SV 108 this value represented ca. 90% (Table 7A).

In this second greenhouse resistance test a few plants from lines SV 108 and SV 98 seemed to be slightly protected against the virus as determined by final height and weight (see Section 3.3.9.), although the average ELISA values were similar to those found in inoculated plants of the controls. With the results obtained here it can be concluded that none of the ORF1/2 transgenic lines tested showed resistance to BWYV.

3.2.6.2. Response of 5’3’S lines to BWYV inoculation

The viral infection rate observed in plants from these lines varied between 70% and 100% as assessed by ELISA. 14 infected plants out of 20 initially challenged with BWYV were found in lines SV 31 and SV 33. For line SV 33, it is assumed that some plants were not infected, although it could be possible as seen from the results of the first greenhouse resistance test that some plants were infected but had low OD_{405} readings. For the other transgenic lines tested ca. 90% to 100% of the initially virus challenged plants were infected with BWYV. In both controls 100% of the plants were infected as determined by ELISA.

3.2.6.2.1. BWYV ELISA

The results of BWYV ELISA in this second greenhouse resistance test showed that all lines tested were susceptible to BWYV. The average OD_{405} readings at 4 wpi of virus inoculated plants of the transgenic and the control lines tested were similar. No major differences were found in the ELISA data at 6 and 8 wpi (Table 6B).

The lowest OD_{405} readings at the final testing time were found in the inoculated plants of lines SV 33 and SV 134 which represented ca. 30% of the average value of both infected controls (Table 6B).

The analysis of individual infected plants of line SV 33 showed that about 50% of the plants had lower ELISA values at 8 wpi than at 4 wpi (average OD_{405} of 0.33). At the same time, two plants of this line had extremely high ELISA titers (an OD_{405} of 3.2). For line SV 134 a similar phenomenon was observed where 11 of the inoculated plants had low ELISA values at 8 wpi (see section 3.3.9.).

3.2.6.2.2. Height

In general, 5’3’S uninoculated plants had an average final height of 52 cm with exception of line SV 88 (44 cm). The uninoculated plants of the two controls reached in average 50 cm (Fig. 8).

Virus infected plants of lines SV 88, SV 135 and both controls had a final growth which was 50% of the respective uninoculated control. On the other hand this value was 60%, 75% and 65% for lines SV 31, SV 33 and SV 134, respectively.

3.2.6.2.3. Final weight

Uninoculated plants of the 5’3’S transgenic and those of the controls had a similar average final weight (12 g). This value was slightly lower for line SV 88 (10 g).

The average final weight of virus infected plants of transgenic lines SV 31, SV 33 and SV 134 was slightly below the average of the uninoculated controls (ca. 70%). Contrarily both infected controls had average weights of 4 g, which represented ca. 30% of the final weight of their respective non-inoculated controls. Similar ratios were calculated for BWYV challenged plants of lines SV 88 and SV 135 (Table 7B).

From the results of this second greenhouse resistance test it can be determined that two 5’3’S transgenic lines, SV 33 and SV 134 were weakly protected against BWYV compared to either both controls or the other three transgenic lines tested. However, this transgene does not confer resistance against the virus, even though in lines SV 33 and SV 134 the average values from height and weight were slightly higher and those of ELISA lower than the ones determined in the infected controls at the end of the experiment.

3.2.6.3. Response of 5'3'AS lines to BWYV inoculation

As assessed by ELISA only 70% and 80% of the initially virus challenged plants of lines SV 28 and SV 77 were infected with BWYV, respectively. Lines SV 86, SV 93, SV 125 and both controls were 100% positive for the virus. Despite the fact that low ELISA values were found in inoculated plants of line SV 77 in the first resistance test, in this second test plants which had escaped infection could be easily detected, since their height at 4 wpi was similar to the one of the non-inoculated plants.

3.2.6.3.1. BWYV ELISA

The results of ELISA at 4 wpi showed that almost all transgenic lines assayed had similar OD_{405} readings to those of the infected controls. These OD_{405} values increased in time, indicating that all lines were susceptible to BWYV. The virus infected plants of line SV 77 tended to keep a constant low average ELISA at all times tested. At the end of the experiment this value was 30% of that observed in the infected non transformed and vector transformed *N. benthamiana* plants. All other transgenic lines tested had average ELISA values similar to the virus infected controls at the end of the experiment (Table 6C).

3.2.6.3.2. Height

The uninoculated transgenic and control *N. benthamiana* plants reached an average final height of 49 cm. Line SV 125 showed an evident decrease in its growth (40 cm), which represented 80% of the average of the other lines tested in parallel (Fig. 9).

The final height of the virus infected plants of all transgenic and control lines tested decreased ca. 60% as compared to the respective uninoculated plants. This was also true for line SV 77, which had low average ELISA values at all sampling times (Fig. 10). BWYV inoculated plants of line SV 93 had a height ratio (infected/uninoculated plants) above the average of the group (ca. 0.7, Fig. 8).

3.2.6.3.3. Final weight

Uninoculated plants of the transgenic lines and of both controls assayed reached an average final weight of 11 g. In this group the lowest weight was determined in plants of line SV 125 (9 g, Table 7C).

In average the final weight of the inoculated plants was ca. 6 g, slightly higher than the one reached by the controls (4 g). All transgenic lines tested showed a weight ratio of infected vs uninoculated plants of ca. 0.5. This ratio was 0.3 in both virus infected controls.

From the data presented above, it could be postulated that line SV 77 had a stronger response to BWYV as determined by the results of ELISA. The ELISA value is ca. 3 times reduced compared to the value found for the infected controls at 8 wpi. However, the final height and weight of the BWYV infected plants of this line were similar to those of the virus infected controls and the other transgenic lines tested in parallel. Although the final height and weight of the virus inoculated plants of line SV 93 were not decreased as those of the other lines tested, the ELISA values were quite high and similar to those observed in both infected controls.

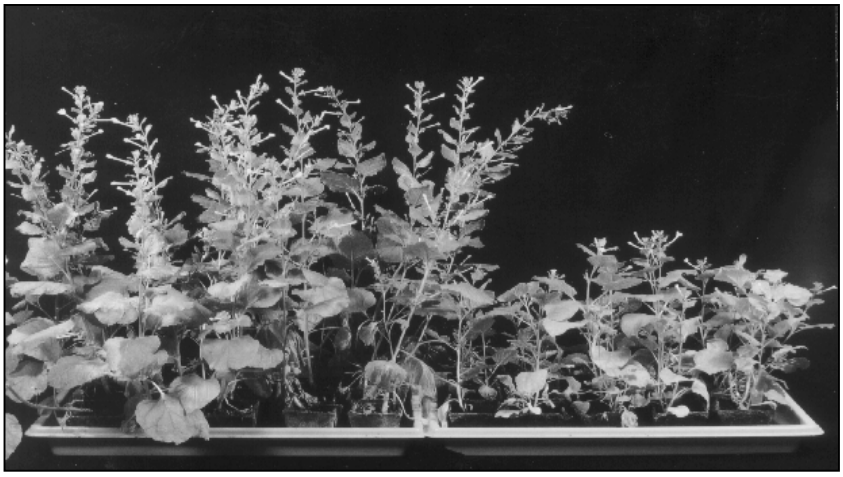


Figure 10. *N. benthamiana* plants from transgenic line SV 77. Healthy plants are shown on the left side, while BWYV infected plants on the right. Although the virus inoculated plants had low ELISA values at all times assayed, the final growth was decreased by ca. 50%, showing no difference when compared to the infected controls.

3.3. Comparison of transgenic plants carrying the same transgene

No major differences in the development of the different transgenic *N. benthamiana* plants of the T1 generation compared to untransformed plants could be observed. However, as noted in section 3.1.6. only 65% of the transgenic plants kept in the glasshouse were able to flower and develop seeds. From these, a few lines (17) were further studied in the two greenhouse resistance tests performed, and some differences in plants carrying the same transgene could be detected.

3.3.1. Transgenic ORF1/2 lines

Uninoculated plants of two of the five ORF1/2 lines analysed reached a diminished final height (SV 110 and SV 112) compared to the other lines tested. This effect was less evident in the second as in the first resistance test.

The virus challenged plants from these two lines also reached the lowest heights at the end of each experiment. The height ratio of infected vs healthy plants was estimated to be 0.2 and 0.4 in the first and second greenhouse resistance test, respectively. This value did not differ from the one found for both controls tested in parallel.

The final weight of the healthy plants of these lines was not as diminished as expected in both experiments. On the other hand virus infected plants had lower weight than the other infected transgenic lines.

When all the data collected from both resistance tests are analysed it can be seen that this two lines had a similar response to BWYV as the controls.

In lines SV 98, SV 108 and SV 117 a large number of plants had low ELISA values at 8 wpi in the first resistance test, while in the second test this phenomenon was observed only for lines SV 98 and SV 108.

3.3.2. Transgenic 5’3’S lines

Non-inoculated and inoculated plants of line SV 88 reached ca. 80% of the final growth of the other transgenic and control plants tested. However, a similar height ratio (infected vs non-infected plants) as that of the controls was determined. The average final weight of the non-infected plants of this line was ca. 75% of the other 5’3’S transgenic lines studied.

The five transgenic lines tested had different responses to viral infection. On the one hand 3 lines, SV 31, SV 88 and SV 135, responded very similar as the controls. On

the other hand, lines SV 33 and SV 134 had a better “tolerance“ to viral infection according to ELISA, height and weight values measured at 8 wpi. BWYV infected plants of line SV 33 had low average OD_{405} readings at the end of both greenhouse resistance tests. For line SV 134 this reduction was more evident in the second as in the first resistance test.

3.3.3. Transgenic 5’3’AS lines

In both greenhouse resistance tests the non-infected and inoculated plants of line SV 125 reached the lowest growth, which represented 80% of the final height of the other 5’3’AS lines. The height ratio of infected vs non-infected plants was similar to the one calculated for both controls. The final average weight of the uninoculated plants was also below the average of the one found for the other lines tested.

In general the average OD_{405} readings at 4 wpi were lower in the first than in the second greenhouse resistance test. However in the first test they tended to increase at 6 and 8 wpi and were very similar to those determined in the second greenhouse resistance test at the same sampling times. At 8 wpi only line SV 77 had in both cases a low average ELISA value. All other 5’3’AS transgenic lines tested reached similar OD_{405} values as those of the infected controls.

3.3.4. Differences between the first and second resistance tests

The transgenic lines analysed should have had a similar response to virus inoculation in both greenhouse resistance tests. However, some differences could be found when comparing some results e.g. final height and weight.

In general, non-infected transformed or untransformed *N. benthamiana* plants had a final height about 1.2 times greater in the first than in the second greenhouse resistance test. A more dramatic effect could be detected on the final weight of these plants. During the second greenhouse resistance test plants reached only 60% of the weight as that in the first test. Nevertheless, the response of the different transgenic lines to BWYV was similar in both resistance tests according to parameters such as the height and weight ratio when compared to those of the controls.

Another significant difference between both experiments was observed in the OD_{405} readings of BWYV ELISA. During the first resistance test the average value of each line was relatively low at 4 wpi, increasing continually up to 8 wpi. However, in the second resistance test high OD_{405} values were initially determined and remained constant during the rest of the experiment (Table 6).

The infection rate of the BWYV challenged transgenic plants was different in both greenhouse resistance tests. Although at least 95% of the challenged control plants were infected with BWYV, only 60% of those of line SV 28 were infected in the second resistance test. The same could be observed with some other transgenic lines, where 60% to 75% of the total BWYV challenged plants had viral titers as assessed by ELISA at 4 wpi.

Apparently the position of the transgenic lines in the greenhouse during the second resistance test was correlated with the efficiency of the transmission of the virus. Transgenic lines which were faced to the outer side of the greenhouse had a relative low number of infected plants (60% to 80%), while those situated near to the inner side of the glasshouse had infection rates ranging from 90% to 100%.

3.3.5. NPTII and BWYV ELISA

The transgenic plants assayed in the two greenhouse resistance test were selected on Km (200 mg/l). Therefore, they should express the NPTII protein encoded within the T-DNA region integrated from the pBin19 vector used for plant transformation.

Due to the variability of the BWYV ELISA observed within each line, it could be thought that some lines might have had some escapes, i.e. plantlets which although grew on Km could happen not to contain the nptII sequence. An NPTII ELISA was carried out to exclude this possibility. For this purpose, leaf samples from two plants of each line having high or low levels of the virus as determined by ELISA at 4 wpi were collected. A sample from a non-infected transgenic plant of each line was also included as control.

All transgenic plants analysed expressed high levels of NPTII protein, indicating that they were transgenic (Table 8). These values did not correlate with the amount of BWYV determined by ELISA, i.e. plants showing low values of NPTII did not necessarily have high BWYV ELISA values. Although samples were adjusted to an equal protein concentration before performing NPTII ELISA, levels of NPTII varied within plants of the same transgenic line.

To confirm that differences in the response to BWYV were not due to loss of the viral sequence in the T1 progeny, genomic DNA was extracted from the same leaf material of the plants analysed by NPTII ELISA and amplified for the viral sequence by PCR. The expected product was detected in all samples tested, excluding any loss of the transgene in these plants (data not shown). No product was amplified from a negative control (genomic DNA of untransformed *N. benthamiana* and water control).

ORF1/2 Lines				5‘3‘S Lines				5‘3‘AS Lines			
Line	Plant	NPTII	BWYV	Line	Plant	NPTII	BWYV	Line	Plant	NPTII	BWYV
98	5	1.50	0.19	31	1	1.64	0.05	28	2	0.59	0.02
	17	1.00	3.18		9	1.45	0.87		7	0.85	1.06
	23	1.51	0.00		23	1.00	0.00		23	1.16	0.00
108	10	1.51	0.10	33	4	1.12	0.00	77	5	0.52	0.01
	19	1.46	1.46		18	1.64	0.77		14	1.54	1.40
	23	1.25	0.00		23	2.88	0.00		23	1.25	0.00
110	3	0.70	0.09	88	9	0.79	0.00	86	6	0.93	0.03
	15	0.74	1.51		15	0.93	0.98		8	0.68	0.88
	23	1.48	0.00		23	1.77	0.00		23	1.34	0.00
112	5	1.52	0.00	134	3	1.05	0.00	93	2	2.89	0.00
	8	0.81	1.21		14	1.24	1.86		6	1.71	0.78
	23	1.29	0.00		23	1.56	0.00		23	1.48	0.00
117	15	1.13	0.24	135	9	0.85	0.98	125	2	0.78	0.00
	19	1.93	2.30		11	1.51	0.05		10	1.20	1.88
	23	1.12	0.00		23	0.99	0.00		23	0.98	0.00
158	3	1.36	0.41	138	3	0.87	0.15	138	3	0.87	0.15
	19	0.94	1.94		15	1.56	1.91		15	1.56	1.91
	23	1.74	0.00		23	1.74	0.00		23	1.74	0.00
Nb	1	0.00	0.39	Nb	1	0.00	0.24	Nb	1	0.00	0.24
	10	0.00	2.67		16	0.00	1.17		16	0.00	1.17
	23	0.00	0.00		23	0.00	0.00		23	0.00	0.00

Table 8 Results of NPTII and BWYV ELISA from infected transgenic plants. To test for possible escapes in some of the transgenic plants used in the greenhouse resistance tests, a single leaf from randomly selected plants, showing either high or low viral infection as assessed by BWYV ELISA at 4 wpi was tested by both assays (results shown correspond to one sample of each case). Plant N° 23 in each line is the respective non infected plant. Lines SV 138 and SV 158 are vector transformed plants. Nb is untransformed *N. benthamiana*.

3.6. BWYV ELISA of root extracts

It should be expected that plants showing a high degree of viral infection according to visual symptoms might have high viral titers. Viral titers of inoculated plants did not necessarily correlate with the “visual” degree of infection in our study. On the one hand, some virus inoculated plants showed a high degree of stunting but had low or middle levels of virus as determined by ELISA (see e.g. Fig. 12, 13 or 14). On the other hand, it could also be observed that BWYV infected transgenic plants which seemed healthy and had “normal” growth and weight compared to their respective healthy controls had high viral titers as assessed by ELISA. Since these differences can be due to the position, size and also part of the leaf taken for virus analysis, a BWYV ELISA assay was carried out with root extract from the virus infected plants.

For this purpose, at the end of the second greenhouse resistance test done with the 5’3’S or 5’3’AS plants roots were tested by ELISA. Pots were allowed to dry and roots were collected, thoroughly washed with water, dried on filter paper and 100 mg were ground in liquid nitrogen and diluted 1:3 with sample buffer (PBS + Tween 20). Samples were incubated overnight at 4°C with the primary antibody and the BWYV ELISA was performed as usual. Roots from non-infected transgenic and non-transgenic plants were included as negative controls. Plates were incubated at 37°C for 40 min. to allow colour development.

The results of this assay were similar to those observed when BWYV titers were determined from leaf sample extracts, although lower OD_{405} readings were found (data not shown). No correlation between levels of virus present in the roots and the degree of viral infection observed by growth parameters was found.

Values of BWYV ELISA from leaves and those observed when using roots as starting material were not correlated. Plants which had a high BWYV ELISA value determined in leaf sample extracts did not necessarily have the highest viral levels detected in the root extracts and vice versa (data not shown). The low ELISA values found when roots were used compared to those of leaves, could be due to at least two factors: i) presence of less virus amount in the roots of infected plants and ii) the method of protein extraction used for roots was not optimal.

3.3.7. Correlation between ELISA and final height

Stunting is a characteristic symptom caused by viral infection and therefore also observed in BWYV infected plants. In this study infected plants showed different degrees of stunting in both resistance tests. No correlation between the data of BWYV ELISA for each infected plant of all the lines tested and the final height reached by each of them could be found. In most cases, high viral titers as assessed by ELISA were present in plants which reached “normal height” and did not show severe viral symptoms. On the other hand, some plants which clearly showed viral symptoms had low OD_{405} readings. In Figures 11, 12 and 13 the ELISA data for each infected plant at 8 wpi is plotted vs its final height (using a logarithmic function for the final height).

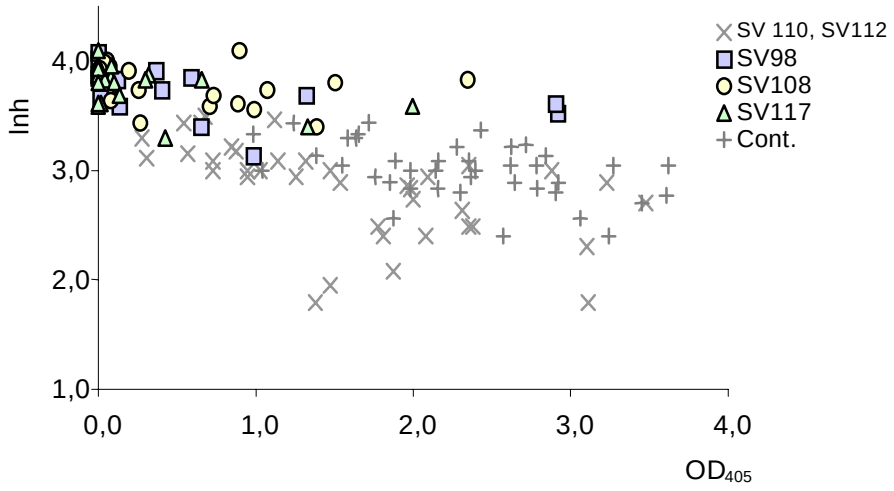
Figure 11 shows the correlation between ELISA values of inoculated ORF1/2 transgenic plants and their final height (as ln) in both resistance tests. Lines SV 110 and SV 112 are shown together, since no major differences with the controls were found. Although inoculated plants of lines SV 98, SV 108 and SV 117 had high OD_{405} readings at the end of the first test, their final height was slightly decreased (Fig 11A). These plants reached final heights which were above the average of the other infected transgenic lines or controls tested, independently of the ELISA value. The results obtained in the second greenhouse resistance test are more dispersed (Fig. 11B), especially for the BWYV inoculated plants of line SV 117. Although the virus infected plants of lines SV 98 and SV 108 had high OD_{405} readings at 8 wpi, their final height was not as depressed as either the infected plants of the controls or those from the other transgenic lines tested. None of the virus inoculated plants of the other two lines studied (SV 110 and SV 112) showed this behaviour. The same is valid for both BWYV infected controls.

The data of plants of the 5’3’S lines are shown in Fig. 12. The results of lines SV 33 and SV 134 are shown separately, while those of lines SV 31, SV 88 and SV 135 are grouped. In general the final height of BWYV infected plants of lines SV 33 and SV 134 was above the one determined for the other lines tested, independent of the OD_{405} at 8 wpi. This effect is more evident in the second than in the first greenhouse resistance test (Fig. 12B). The other lines tested (SV 31, SV 88 and SV 125) behaved similarly as the controls reaching final heights which were ca. 50% of the uninoculated controls.

Fig. 13 shows the data of the inoculated plants from the 5’3’AS lines tested. In this case, as mentioned above all lines responded as controls to BWYV inoculation. Although most of the virus infected plants of line SV 77 always had low ELISA values (ranging from 0.1 to 0.6) their final height was similar to the one observed in the other 5’3’AS inoculated plants and controls tested (Fig. 13). Interestingly, these values represented an OD_{405} reduction of 4 and 3 times compared to the average of the infected controls in the first and second greenhouse resistance test, respectively. In general, the final height reached by the infected plants from this line was ca. 65% of that of the uninoculated plants and the average weight of these plants was ca. 40% of the noninfected controls.

In part this phenomenon could be due to the sampling of the different inoculated plants, where leaves can contain different amounts of virus. However, this was apparently not the case since leaf discs from three different levels of the plant were taken for each sampling. There were only few exceptions where this could not done, where due to a strong viral infection, plants had very little amounts of leaf material.

A



B

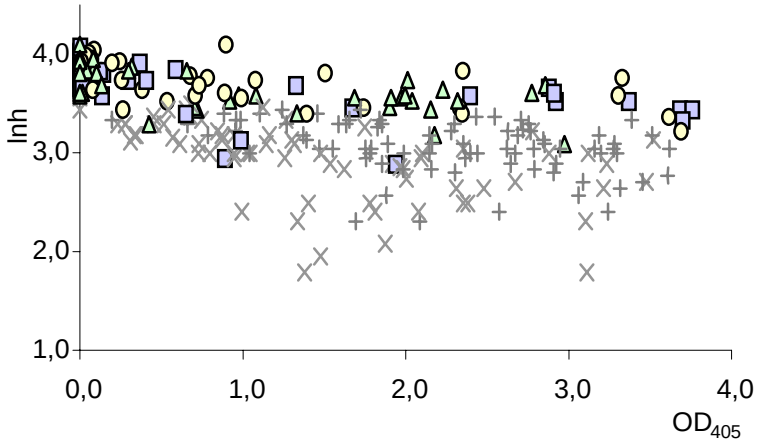


Figure 11 Correlation between the OD_{405} (at 8 wpi) and final height (as ln) of ORF1/2 infected plants. In A and B the results from the first and second greenhouse resistance test are shown, respectively. Note that lines SV 98, SV 108 and SV 117 have relative low ELISA values and higher heights than the rest of the lines tested. In the second resistance test the ELISA data are more disperse. Controls (Cont) included were the vector transformed line SV 158 and untransformed *N. benthamiana* plants.

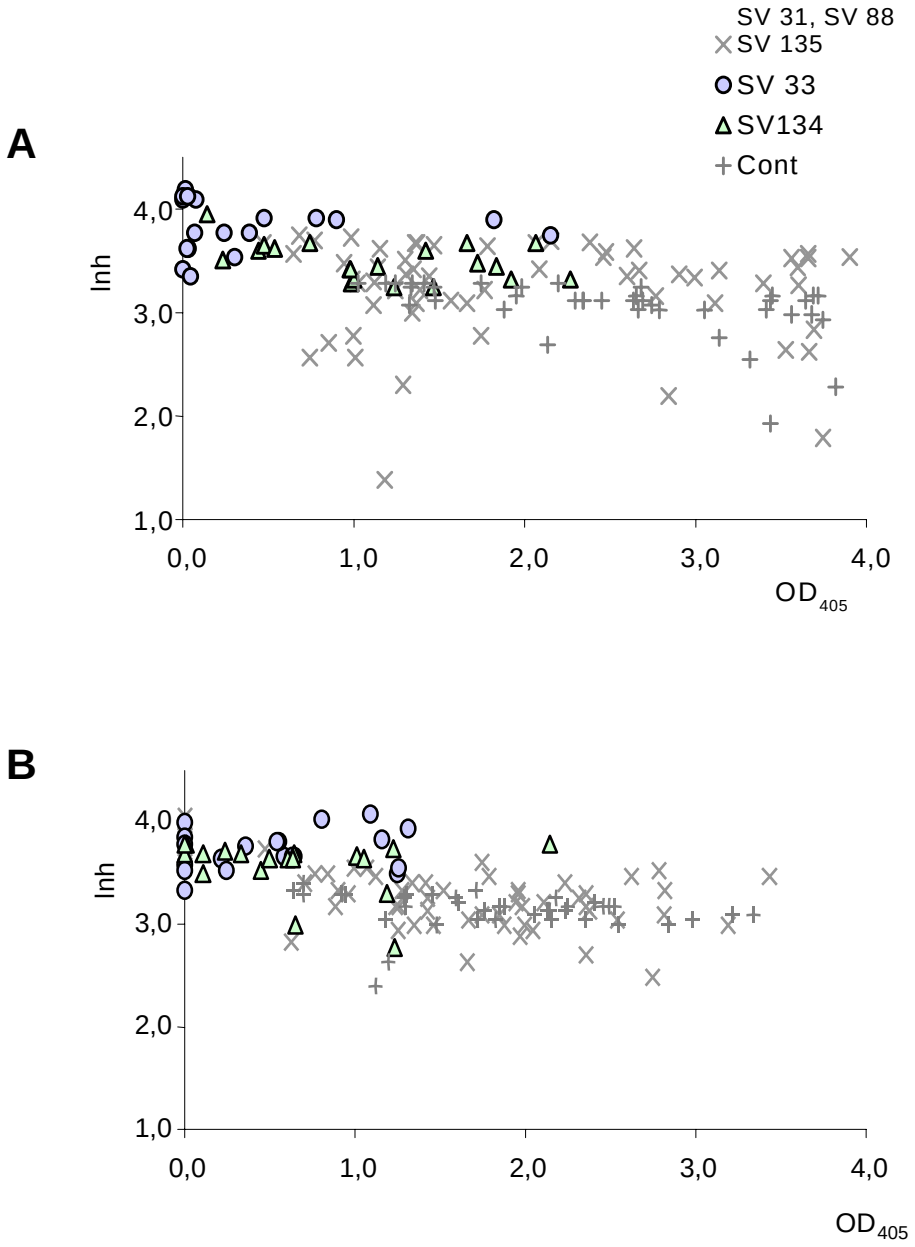


Figure 12 Correlation between ELISA values (at 8 wpi) and final height (as ln) of 5‘3‘S lines. A and B show the results from the first and second greenhouse resistance tests, respectively. Note that in both cases line SV 33 reached high final heights and low ELISA values, compared to the inoculated controls. Controls (Cont) included were a vector transformed line (SV 138) and untransformed *N. benthamiana* plants.

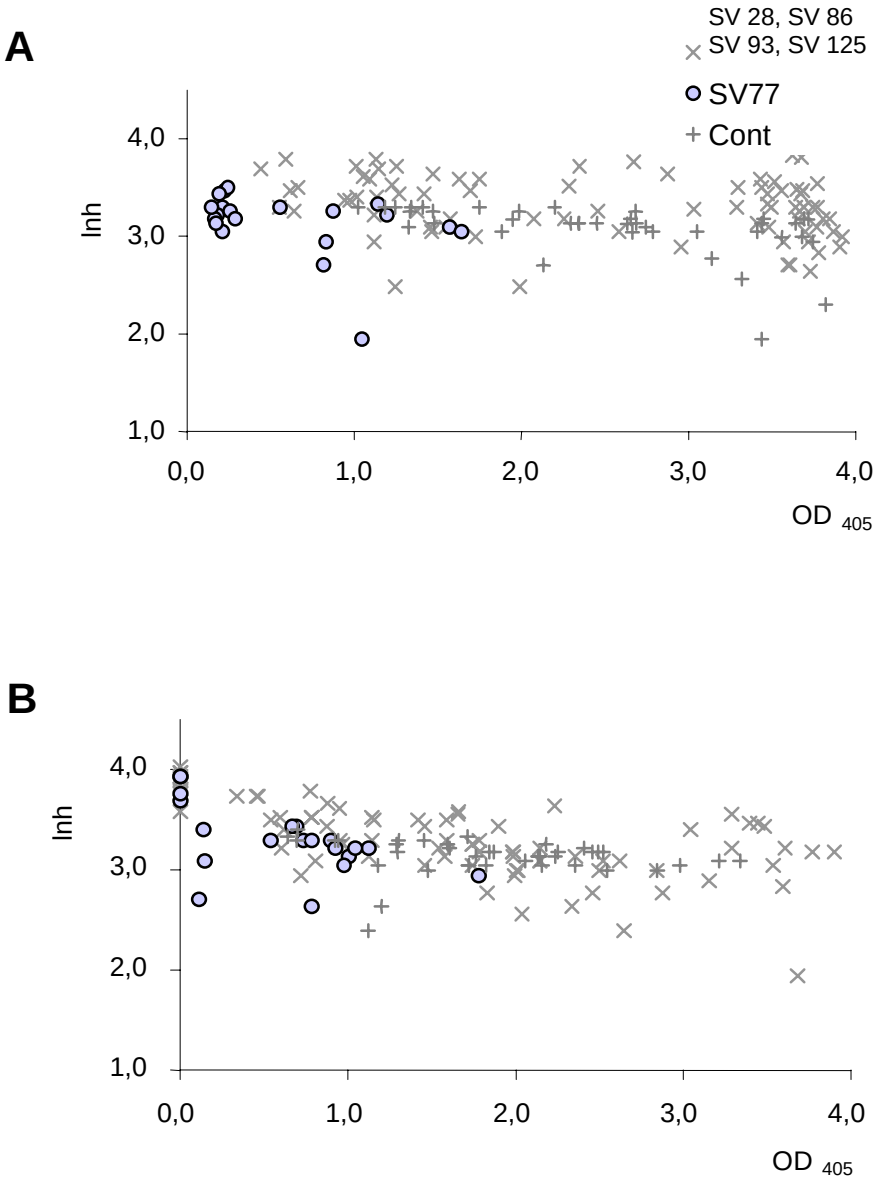


Figure 13 Correlation between the OD_{405} readings (at 8 wpi) and the final height (as ln) of BWYV infected plants of the 5‘3‘AS lines tested. A and B show the results obtained in the first and second greenhouse resistance test, respectively. Note that line SV 77 had relatively low ELISA values in both greenhouse resistance tests, but the final height was similar to the other lines tested. The controls (Cont) included were a vector transformed line (SV 138) and untransformed *N. benthamiana*.

3.3.8. Resistance test of transgenic plants not selected on Km

The selection on kanamycin of transgenic plantlets of the lines tested for resistance in the greenhouse could have had an effect on their response to virus inoculation. To study this possibility, seeds of two ORF1/2 lines were planted directly on soil. Lines SV 112 and SV 117 were choosen, since they had an opposite response to viral inoculation in the first greenhouse resistance test. Once seeds had germinated, two sets of 10 plantlets from each line were prepared, one was infected with BWYV and the other kept as healthy control.

Before inoculating plants with the virus, genomic DNA was extracted from each plant and the viral specific sequence was amplified by PCR. As a negative control genomic DNA extracted from untransformed *N. benthamiana* was used. In all cases 7 out of the 10 plants analysed of each set contained the viral sequence. No product amplification could be detected in the negative control (Fig. 14).

The resistance test was performed as described in Materials and Methods. Briefly, 5 to 7 green peach aphids were transferred to newly emerging leaves of the *N. benthamiana* plants, allowed to feed for 3 days and then eliminated by applying an insecticide. A BWYV ELISA from leaf samples was performed at 4, 6 and 8 wpi. At these same sampling times, height was measured and the weight was determined at 8 wpi. This study was carried out in parallel to the second ORF1/2 greenhouse resistance test.

Plants of line SV 112 germinated and grew faster than those of line SV 117. However, at 8 wpi the non-infected plants of line SV 117 had developed better than those of line SV 112 reaching a higher final average weight and height. This result is similar to the one observed when plantlets from these lines were selected with Km.

As tested by ELISA at 4 wpi, the BWYV challenged plants of both lines were positive for the virus. The infected plants of line SV 117 showed a stronger response to viral inoculation than line SV 112, according to ELISA values as well as to parameters of final height and weight (Table 9). These results were similar to those observed in the resistance test carried out with Km selected plantlets.

Therefore these two transgenic lines were not resistant against BWYV. However, it could be determined that kanamycin selection of plantlets did not affect the development and further response of the different transgenic lines to virus inoculation.

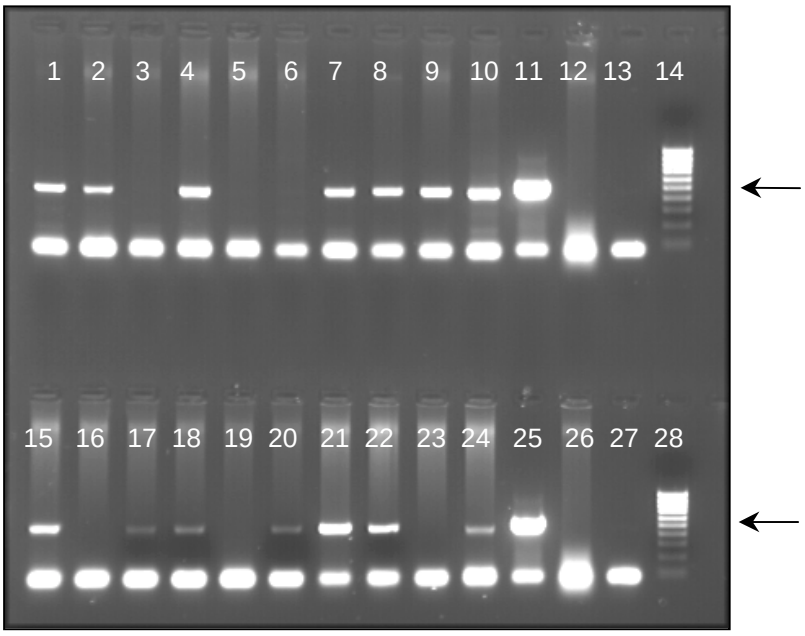


Figure 14 PCR products of ORF1/2 lines SV 112 and SV 117. Seeds from both lines were planted directly on soil and allowed to germinate in the greenhouse. Genomic DNA was extracted from each plant and tested by PCR for the presence of the transgene. In both cases 7 from the 10 plants tested were positive. Lanes 1-10 and 15-24 show the results of lines SV 117 and SV 112, respectively. Lanes 11 and 25 correspond to the positive control (plasmid DNA), lane 12 and 26 to untransformed *N. benthamiana* and lanes 13 and 27 to the water control. The arrows indicate the expected product of ca. 500 bp.

Line	ELISA 4wpi	ELISA 6wpi	ELISA 8wpi	hinf	hcon	winf	wcon
112	0.67 ± 0.60	1.25 ± 0.85	1.39 ± 0.99	22.6 ± 8.0	36.0 ± 7.8	2.4 ± 2.4	8.1 ± 2.5
117	0.65 ± 0.53	0.55 ± 0.53	0.56 ± 0.51	27.7 ± 10	43.3 ± 3.5	6.9 ± 5.3	8.4 ± 3.8
158	1.15 ± 0.61	1.86 ± 0.28	2.18 ± 0.59	22.2 ± 4.8	53.4 ± 8.7	3.4 ± 1.1	13.0 ± 5.1
Nb	1.11 ± 0.62	2.14 ± 0.87	2.20 ± 0.86	26.6 ± 3.9	54.1 ± 3.7	5.0 ± 1.9	11.5 ±3.3

Table 9 Results of the greenhouse resistance test performed with transgenic plants not selected on Km. Seeds of two ORF1/2 lines (SV 112 and SV 117) were planted directly on pots. Ten plants from each line were either inoculated with BWYV or used as healthy controls. Leaf samples were taken at 4, 6 and 8 wpi for ELISA. The height (hinf and hcont, for inoculated and noninoculated plants, respectively) was determined at these same sampling times, while the weight (winf and wcont, for inoculated and noninoculated plants, respectively) was measured at 8 wpi. The results shown are the average of 7 transgenic plants (as tested by PCR), in each case.

3.3.9. Analysis of individual plants of transgenic lines with low ELISA values

Although the results of both resistance tests showed that none of the transgenic lines tested was resistant to BWYV, in some lines a few plants seemed to have some protection against the virus. This protection was determined since in general these plants had values which were lower than (ELISA) or higher than (height and weight) those found for the infected controls. Therefore, each of these parameters was analysed for each plant, especially the values of ELISA at 8 wpi, where an OD_{405} range from 0.1 to 0.5 was defined as low. The data of BWYV inoculated plants which had ELISA in this range were selected and analysed. This was especially notable in lines SV 33, SV 98, SV 108, SV 117 and SV 134 which had a large number of plants with this behaviour. A new average for final height, weight and ELISA was calculated for each of these lines (Table 10). In some cases differences in the average final height and weight of plants with low OD_{405} compared to the respective lines including the 20 inoculated plants were found.

Line	N°	Height	Weight	ELISA
33	16	51.5 ± 9.4	13.8 ± 9.7	0.09 ± 0.09
	7	43.0 ± 4.9	10.0 ± 2.9	0.34 ± 0.07
134	9	36.7 ± 5.3	8.0 ± 3.8	0.36 ± 0.10
	11	36.9 ± 4.3	8.4 ± 2.2	0.42 ± 0.18
98	14	45.0 ± 5.2	17.6 ± 6.2	0.12 ± 0.11
	11	45.0 ± 3.3	7.2 ± 1.9	0.05 ± 0.07
108	12	44.0 ± 7.0	17.8 ± 8.0	0.23 ± 0.19
	13	49.0 ± 5.9	11.0 ± 3.0	0.21 ± 0.24
117	17	45.0 ± 5.9	12.2 ± 4.4	0.08 ± 0.08
	2	45.0 ± 1.0	11.2 ± 0.7	0.03 ± 0.03

Table 10 Inoculated transgenic plants with low ELISA values. The data of infected plants having final low ELISA values (from 0.1 to 0.5) are shown. Results are shown for lines which had a large number of plants with this behaviour in both resistance tests. The average of height, weight and ELISA at 8 wpi is shown in each case. The upper and lower row show the average of the first and second greenhouse resistance test, respectively. N° indicates the number of plants with low ELISA for each transgenic line.

3.3.9.1. Line SV 33

Of the five 5'3'S transgenic lines analysed line SV 33 had a stronger response to BWYV inoculation than the controls. In this case 23 of the total infected plants in both resistance tests had low ELISA values at 8 wpi (Table 10). In the first experiment the OD_{405} was ca. 25 times less than the average of the infected controls, while in the second resistance test it represented a 5 times reduction. It is also interesting, that in the first experiment almost all plants tested (80%) had low final OD_{405}, while in the second test only 50% of the plants which were assumed to be infected (14) showed this behaviour. The final height of these plants was above the average determined for all plants of this line (Fig. 8). This value represented ca. 80% of the final average height of the uninoculated control. However, the analysis of these individual plants showed that some had similar final heights as the average of the respective uninoculated controls (Figures 12 and 15).

3.3.9.2. Line SV 134

Twenty of the total infected plants in both resistance tests showed a stronger response to viral infection (Table 10). Most of the SV 134 inoculated plants which had low ELISA values were tested in the second greenhouse resistance test.

A 4 times reduction in the ELISA of these plants compared to the infected controls was found in both resistance tests.

No difference in the final height between plants with low ELISA values and the average height of all plants of this lines were found. The final height of the BWYV challenged plants was in average 65% of the respective uninoculated controls in the first resistance test, therefore their response to virus inoculation was weaker as the one observed in line SV 33 (Fig. 12 and 15). However, in the second greenhouse resistance test a few infected plants of line SV 134 reached final height similar to the average of the uninoculated control.

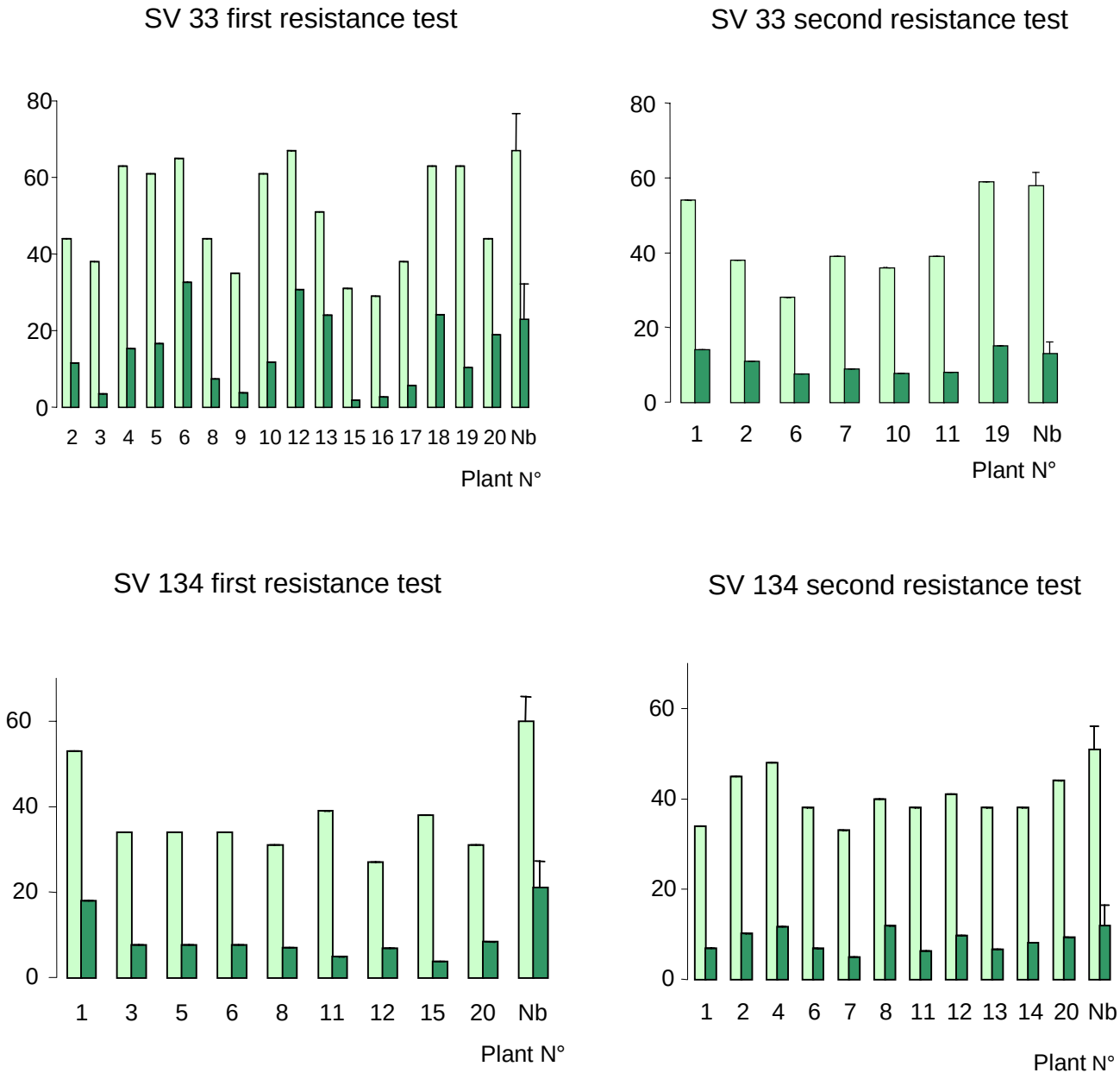


Figure 15 Infected plants with low OD_{405} values of the two 5‘3‘S lines. Each bar represents a single plant, the height (■) and weight (□) measured at the end of the resistance tests (8 wpi) are shown in each case. Nb represents the average final height and weight for the corresponding uninoculated control of each transgenic line (20 plants in each case). The height is represented in cm, while weight in g.

3.3.9.3. Line SV 108

12 and 13 of the inoculated plants of this line had a weak protection against the virus in the first and second greenhouse resistance test, respectively. When plants were grouped according to the final OD_{405} readings a ca. 10 times reduction in the average ELISA values compared to those of the infected controls in both resistance tests was found (Tables 6 and 10). In both experiments some plants had lower ELISA value at 8 wpi than at 4 wpi.

Plants which had low ELISA in average reached a final height which represented ca. 70% of the one observed in the respective healthy controls during the first resistance test. Interestingly, in the second test most of the BWYV inoculated plants had final heights similar to the average of the uninoculated group (Fig. 11 and 16). Therefore, this line seemed to have a weak protection against BWYV. If plants which had low ELISA values in the first test are considered individually, it can be seen that some reached similar final height and weight as the average of the uninoculated control (Fig. 16).

3.3.9.4. Line SV 98

Twenty five BWYV challenged plants from this line could be grouped as having low ELISA values. A ca. 25 reduction in ELISA compared to the infected controls in both resistance tests was determined. This value is quite high due to the extremely low OD_{405} of these plants at 8 wpi. In average the plants with low ELISA reached a final height of 75% and 85% of the respective uninoculated controls in the first and second resistance test, respectively (Fig. 16).

3.3.9.5. Line SV 117

Virus inoculated plants from this line showed an extremely opposite response to BWYV in the first and second resistance test. In total 17 plants of this line in the first test had initially high OD_{405} values which decreased at 8 wpi (data not shown). This represented a 25 time reduction compared to the ELISA values of the infected controls tested in parallel. The final average height of plants in this group represented 70% of that of the uninoculated control, however a few plants had similar height as the noninfected control (Fig 11). However, in the second greenhouse resistance test only 2 plants had low ELISA values at the end of resistance test, which could have been escapes.

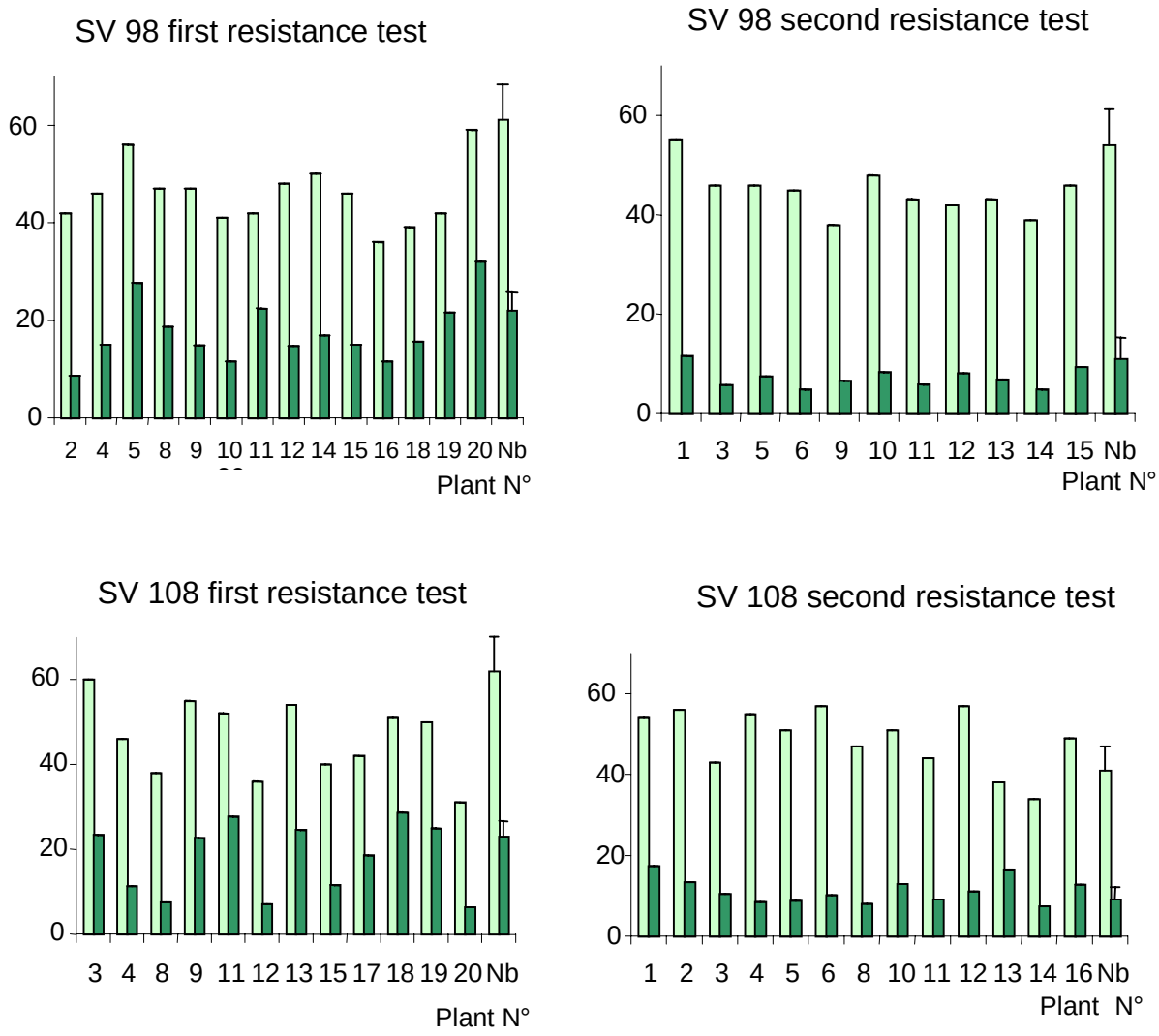


Figure 16 BWYV infected plants of ORF1/2 lines that had low ELISA values. In each case plants with low ELISA values of these lines are shown. Each bar represents the final height (■) and weight (□) determined at 8 wpi of a single plant. Nb is the non-infected control of each line, in this case the average final height and weight of 20 healthy plants is shown. The height is represented in cm, while the weight in g.

In summary of all the ORF1/2 and 5’3’S transgenic plants tested, ca. 50 BWYV infected plants per construct seemed to have a certain degree of “tolerance” against the virus (see Figures 11, 12, 15 and 16). Even though in some cases they had “relative high” viral titres as assessed by BWYV ELISA, their development was normal reaching in most cases ca. 75% and 80% of the average final height and weight of the uninoculated plants, respectively.

A few virus inoculated plants of some of the transgenic lines tested were not infected by the virus as assessed by ELISA in the second resistance test. This can be concluded, since the same lines were completely susceptible to BWYV when analysed in the first greenhouse resistance test. As well, those plants that had escaped infection reached final height and weight similar to the average of the uninoculated plants. It must be kept in mind that BWYV is only transmitted by aphids and it can not be mechanically inoculated as done with many other plant viruses.

An interesting result was seen in the analyses of “infected” plants of line SV 33, which in some cases had low ELISA values at 4 wpi. This value increased at 6 wpi decreasing thereafter, where in some cases it reached background OD_{405} readings. Therefore, it is difficult to determine if this low value was due to escapes, “low initial virus inocula”, or if these plants could actually “tolerate” a higher level of virus before showing symptoms and becoming infected. In the second resistance test, the same phenomenon was observed, although in this case it was assumed that the infection rate was lower (75%).

4. DISCUSSION

In the present study *Nicotiana benthamiana* plants were transformed with three different viral transgenes derived from BWYV, ORF1/2, 5'3'AS and 5'3'S. We tested the response of plants challenged with BWYV by means of green peach aphids.

The results clearly showed that none of the 15 transgenic lines tested was resistant to viral infection. The sequence encoding the 5'3'AS did not yield resistant plants. Some transformed plants of 5 transgenic lines containing either the 5'3'S or the ORF1/2 sequences seemed to have a weak protection against BWYV. The virus amount in these plants was significantly reduced compared to the infected controls as assessed by ELISA. The final height and weight were comparable to the uninoculated controls.

4.1. PLANT TRANSFORMATION AND CHARACTERISATION

The transformation protocol employed enabled the regeneration of a large number of *N. benthamiana* plants (ca. 120), of which at least 90% contained the corresponding viral insert and expressed the nptII gene as assayed by PCR and NPTII ELISA, respectively. In a few NPTII positive plants the viral insert could not be detected, it could be possible that this sequence was deleted during the transformation process, either before or during transfer of the T-DNA from the bacteria to the plant cell (Gheysen et al., 1990).

Although the transgenic plants developed similarly to non transformed *N. benthamiana* some did not flower generating no seeds, while others did flower but generated very little amounts of seeds. As well seeds obtained from ca. 30% of the T1 generation did not germinate or had a very poor growth when tested for segregation on kanamycin. According to Brederode et al. (1995) the low germination rate can be explained by the transformation procedure itself and is not specific to a particular gene construct. On the other hand, Brunetti et al. (1997) have found that the anormal phenotype observed in transformed tomatoes could be due to a toxic effect of the viral insert. Interestingly, when they firstly transformed *N. benthamiana* plants with the same construct no toxic effect could be observed, but a large proportion of the transformed plants did not flower or their seeds had a very poor germination when selected on kanamycin. Other authors (reviewed by Gheysen et al., 1998) have described that silencing could be responsible for the low germination observed in seeds of some transgenic lines. This phenomenon is especially evident when a large number of copies of the T-DNA are inserted in the plant genome, this silencing effect can be observed in the transformed plant, but as well when it ages or in its seeds.

As mentioned above 70 of the total transgenic plants produced generated seeds in enough amounts and that were able to grow at 200 mg/l of Km. In order to test some lines for resistance against BWYV, other criteria were analysed such as levels of NPTII of To, Km segregation tests, normal phenotype and results of PCR. Only plants which fulfilled these parameters (ca. 60) were selected for further study.

Due to the impossibility to analyse all these lines a limited number of 5 lines per construct were randomly selected. Therefore, only 15 transgenic lines were assayed in the greenhouse for resistance against BWYV.

In both resistance tests the final height of the uninoculated plants of lines SV 88, SV 125, SV 110 and SV 112 was below the average of the other transgenic and control lines tested (by ca. 20%). There was no significant variation intra or intergroup in the height and weight of uninoculated plants of the other lines tested. On the other hand, evident differences in the final height and weight of inoculated plants of all the transgenic lines tested for resistance were found.

All uninoculated controls tested in the second greenhouse resistance test reached a lower height and weight compared to the first resistance test. In part this can be explained by the longer day length during the first (summer) than in the second greenhouse resistance test (late summer). The external temperature should have not played a major role, since plants were kept in the glasshouse under controlled temperature during both resistance tests. The total amount of light in the greenhouse was increased during the second test, in order to simulate the conditions found in summer. In general the differences due to the seasons in which the experiments were carried out did not affect the response to BWYV of most of the transgenic lines. However it can be possible that plants of a few lines (e.g. SV 117 and SV 134) responded to BWYV differently due to environmental conditions as described in other studies (Brederode et al., 1995).

PCR or NPTII ELISA performed from randomly selected T2 plants of the transgenic lines assayed in the resistance tests confirmed that none showed either loss of the viral sequence or low expression of the NPTII protein.

The results of Northern blot showed that some ORF1/2 lines which had some degree of protection against BWYV expressed different levels of the transgene. Lines SV 98 and SV 108 showed a weak protection against the virus in both resistance assays, while for line SV 117 this low degree of resistance was only observed in the first resistance test. Line SV 98 had high levels of mRNA of the viral sequence, while lines SV 117 and SV 108 expressed middle and low levels, respectively. Similar results were found when the expression of the nptII gene was analysed in these lines. When tested on Km (200 mg/l) lines SV 98 and SV 108 had 100% of germination, therefore it seems likely that they had integrated more than three copies

of the transgene. On the other hand lines containing a single copy responded similarly to the susceptible controls (SV 110 and SV 112) and had high levels of expression of the transgene and nptII gene. Therefore it can be possible that for line SV 108 cosuppression is taking part in the response of this line to virus inoculation.

In the 5'3'S lines tested SV 33, which had a stronger response against the virus than the controls, high levels of expression of the transgene were detected by Northern blot. Lines which were susceptible to the virus had as well high levels of the transgene RNA. Therefore in this case it is not probable that cosupression is involved in the response to the virus. Although line SV 134 had a weaker degree of protection to the virus than line SV 33, no mRNA of the transgene could be detected by Northern blot. The results of Km segregation of these two lines indicated that lines SV 33 and SV 134 integrated more than 3 and 1 copy(ies) of the transgene, respectively.

A rep-specific antibody was not available, therefore expression of the transgene was characterized only at the RNA level using Northern blot hybridization assays. Prüfer et al. (1999) have raised mono- and polyclonal antibodies against P1 of PLRV (encoded by ORF1), but they were unsuccessful to obtain an antibody against the P1/P2 protein (encoded by ORF1/2).

There seemed to be a tendency of lines which had integrated more than 3 copies of the transgene to have a weak protection against the virus. This was not true for the 5'3' AS line SV 86. It is possible that in this case the construct itself does not confer resistance since it has the first 400 bp of the 5' end of the viral genome in antisense orientation. In general no correlation between the levels of RNA expression and degree of protection was found, except for line SV 108.

A lack of correlation between the degree of resistance and the expression level of the transgene has been observed in many reports (Sinisterra, 1999; Braun et Hemenway, 1992; Longstaff et al., 1993; Silva-Rosales et al., 1994). In these cases, the type of resistance has been referred to as RNA-mediated. It is also characterized by a lack of dependence upon inoculum dose and a narrow spectrum of protection, often associated with the presence of multiple copies of the transgene or transgene tandem repeats. Longstaff et al. (1997) have postulated that this lack of correlation can be due to variation in the cell specificity or timing of expression of transgenes. As observed by Noris et al. (1996), when using C1 TYLCV transformed *N. benthamiana* plants, the relatively low level of resistance obtained in the transgenic lines may indicate that the expression of the transgene in plants is problematic and there may be a selection for cells that do not express the transgene or do so at low levels.

4.2. GREENHOUSE RESISTANCE TESTS

Mechanical inoculation is commonly used in greenhouse resistance tests to challenge transgenic plants with virus, since a known amount of the virus can be inoculated to each plant. This rarely occurs in the fields, where normally viruses transmitted by vectors and in most cases as a mixed infection (Matthews, 1992). The method of inoculation used to infect *N. benthamiana* plants in our experiments is similar to the way in which the virus infects plants in the fields. In the case of aphid transmitted viruses it is always difficult to measure the efficiency and initial virus amount transmitted to plants and therefore to know if a single plant was infected. In most studies it is assumed that when the controls show 100% of infection, the viral transmission was successful for the transgenic lines tested in parallel.

4.2.1. BWYV ELISA

The amount of luteoviruses has been reported to vary among different leaves on the same plant (Pereira and Lister, 1989; Mowry, 1995). This effect was minimized in our study by taking leaf discs from three different levels of each BWYV inoculated plant. Despite this sampling method, a large variability in the data from BWYV ELISA was found within each line assayed, which was less evident in the infected controls. This variability has also been observed in other studies with luteoviruses, where a considerable variation in OD_{405} from plant to plant was found (Presting et al., 1995; Bruyère et al., 1997; Graham et al., 1997). This may reflect differences in the amount of inoculum originally delivered by aphids and/or differences in the rate of spread of the virus within the plant from the initial site(s) of infection (Bruyère et al., 1997). It can as well be assumed that slight differences in the physiology of plantlets during or following inoculation may play an important role (Presting et al., 1995).

The OD_{405} values obtained from the BWYV ELISA performed at 4, 6 and 8 wpi tended to increase in time especially in those lines which were completely susceptible to viral infection (see Table 6).

In the literature a few examples of correlation between ELISA and damage found in inoculated plants has been described. Presting *et al*. (1995) transformed potatoes with the CP of PLRV and challenged them with the virus using green peach aphids. They found that the PLRV titer as assayed by ELISA was reflected by plant appearance. The authors assumed that the high light intensity and warm temperatures employed in the greenhouse promoted symptom expression. Tenllado et al. (1996) have found that virus accumulation on inoculated and upper leaf tissue, as determined by DAS ELISA, correlated with visual symptoms in the different 54-kDa PMMV transgenic *N. benthamiana* plants when assayed in greenhouse resistance tests.

The levels of virus detected in our study was not always correlated with the degree of infection found in plants. Ponz and Bruening (1986) have described that low levels of some viruses can cause severe damage in plants, and as well some tolerant cultivars can suppress symptom formation without reducing viral replication. Some transgenic plants had high ELISA values in our study, therefore classified as susceptible showed mild or no symptoms due to BWYV infection, i.e. had a tolerant response. In general they reached final weight and height similar to the average of their respective healthy control. Most of the inoculated plants which showed this response corresponded to lines SV 33, SV 98, SV 108, SV 134 in both experiments and SV 117 in the first greenhouse resistance test. In these lines a large number of plants were apparently “normal”, even though they had variable ELISA values (see Fig. 11 and 12). On the other hand some infected plants which had low OD_{405} were sensitive to the virus reaching depressed final height and weight, and showing typical BWYV symptoms. This was clearly observed in plants of line SV 77 in both experiments performed (Fig. 13).

The lack of correlation between the OD_{405} values and the degree of infection in plants found in our study indicates that the ELISA data must be carefully interpreted, since a low OD_{405} value does not necessarily imply resistance or tolerance of a plant against the virus.

4.2.2 Rate of infection of transgenic lines

To determine the rate of infection in the transgenic lines most authors assume that if the controls are 100% infected, then the transgenic lines must have a similar rate of infection. If after inoculation any uninfected transformed plant is found it is unlikely that it could have escaped inoculation (Noris et al., 1996). A similar assumption has been made by Kawchuk et al. (1990) when inoculating CP-PLRV transformed potatoes with PLRV using 5 aphids per plant, the number of escapes in the transgenic lines should be similar to those observed in the virus inoculated controls. In our study 100% of the controls were infected with the virus, only in one case a single plant escaped infection (untransformed control, in the second greenhouse resistance test). Therefore we should assume that no escapes occurred in the inoculated transgenic lines, however this was not the case. In the second greenhouse resistance test 95% to 100% of the susceptible plants were infected by BWYV, but the rate of infected plants in some transgenic lines was only 70% (e.g. SV 28, SV 77). To calculate the rate of infection it was assumed that inoculated plants which had an OD_{405} value below 0.1 were not infected by BWYV.

In our study the low infection rate can be explained by different factors, among them:

a) The time of the day when aphids are transferred to plants has a great influence on the viral transmission efficiency (Johnstone et al., 1984). In both greenhouse resistance tests performed, *M. persicae* was transferred early in the morning. As well the environmental temperature has been described to affect the rate of reproduction of aphids (El Din, 1976). In the second test it could be possible to have slightly lower temperatures in the greenhouse than in the first resistance test.

b) The amount of light available during the acquisition access period (AAP) of aphids is also a critical point on virus transmission and further symptom development in plants (Johnstone et al., 1984; Gielen et al., 1996). This factor could have played an important role during the second resistance test. The first greenhouse resistance test was carried out during summer, while the second resistance test was performed in late summer, therefore the amount of sunlight available was diminished. Although artificial light was supplied in the latter case it may not be comparable to the intensity of natural sunlight. Presting et al. (1995) have observed that differences in the day length regimes when resistance experiment were performed affected the results with CP PLRV transformed potatoes.

c) The distribution of the transgenic lines in the greenhouse seemed also to have an influence on the low infection rate, since plants that faced to the outer side of the glasshouse had a lower infection rate than those placed in the inner side. It is possible that the light supplied in the greenhouse had a better effect on plants located on the internal than on the outer side. In part this could also be explained by the availability of light for the aphids and plants in the second greenhouse test.

d) It could be possible that aphids which were placed on the transgenic plants had low amounts of virus or were unable to transmit it, while those transferred to the susceptible lines were more effective. Stevens et al. (1995) found that some aphids which contained BWYV, as assessed by ELISA, did not transmit the virus to indicator plants, assuming that it could be due to damage of aphids during handling.

e) Plants were inoculated with low amounts of virus. Unfortunately it is not possible to measure the amount of virus initially inoculated in a single plant or to inoculate all plants with the same initial amount of BWYV, as done with mechanically transmitted viruses. Some lines could have resistance to low levels of virus. In some replicase mediated protection studies it has been demonstrated that some lines tolerate or show resistance to very low virus inoculation (Guo and Garcia, 1997; Brederode et al., 1995).

The greenhouse resistance tests were performed at least 4 times independently one from each other and in all cases all susceptible plants were infected as assessed by ELISA. However any of the factors mentioned above can not be ruled out to explain the low infection rate determined in the transgenic plants in the second greenhouse test.

Some BWYV inoculated plants of transgenic lines SV 33, SV 77, SV 98, SV 108, and SV 134 had low OD_{405} during the entire experiment in both experiments. In general we considered that these plants were not resistant against the virus, because firstly the virus was able to replicate in the cell and secondly, the possibility that plants had escaped infection can not be ruled out. In the second resistance test challenged plants of line SV 77 that had escaped virus inoculation, were easy to detect since they reached similar heights as plants of the healthy controls. However in other lines a few infected plants (as determined by ELISA) reached similar final height and weight as the healthy controls (e.g. in lines SV 33 and SV 108). In this case it was difficult to define if a plant with low ELISA had escaped viral inoculation or actually was infected with BWYV. Similar results were observed by Presting et al. (1995) who found large variations in ELISA readings in some potato “resistant” lines transformed with the CP of PLRV. In this case they could not differentiate if plants with low ELISA corresponded to escapes or infected “resistant” plants.

Therefore, in future studies a higher number of aphids per plant should be employed to assure a good level of viral infection. However it must be kept in mind that firstly, this would not represent what actually occurs in the field, since plants would be submitted to a high viral pressure and secondly, it could mask a resistance response shown by some transgenic lines. As well the viral strain of BWYV used in this study is a highly infective and aggressive strain compared to those found in the fields. Other clones of *M. persicae* could be tested for their virus transmission ability. Bourdin et al. (1998) used 15 different clones of *M. persicae* and 2 of *M. nicotianae* to study the rate of transmission of two isolates of PLRV. Their results showed that one of the isolate tested had transmission rates that ranged from 0% to 71%. The authors suggested that the transmission process and its specificity depend on close relationships between aphid clones and virus isolates. Schliephake et al. (2000) studied the transmission of BMYV and BWYV by 24 different aphids. They have demonstrated that *M. persicae* is the main vector which transmits BWYV (96,4%), while *M. nicotianae* is able to transmit the virus only in 8% of the cases.

4.2.3. RESPONSE OF TRANSGENIC LINES TO VIRUS INOCULATION

Three different types of responses were observed in our study for *N. benthamiana* plants transformed with ORF1/2, 5'3'AS and 5'3'S when challenged with BWYV:

a) completely susceptible lines (SV 28, SV 31, SV 86, SV 88, SV 93, SV 125, SV 135, SV 110 and SV 112);

b) lines with low ELISA titers, but showing viral symptomatology (SV 77);

c) lines with low ELISA titers and not depressed physical parameters (lines SV 33, SV 98, SV 108 and SV 134 in both assays and line SV 117 in the first resistance test).

Since lines which were susceptible to BWYV are of no interest for this study they will not be further discussed.

According to the data of final height and weight of the inoculated plants of line SV 77 in both resistance test it could be defined as susceptible to BWYV. However, a large number of plants (ca. 70%) had low OD_{405} values during the experiment, which represented a 4 times reduction compared to the controls. The same could be observed in lines SV 110 and SV 112, but only with the ELISA data at 4 and 6 wpi in both resistance tests. In the second resistance test these two lines showed a ca. 2 times final reduction in ELISA compared to the inoculated controls. These reduced OD_{405} values might reflect an inhibition of viral replication, but since all plants were stunted, it can be clearly deduced that BWYV replicated in the cells. Despite these results, it is not proposed that any of these lines is resistant to BWYV inoculation. In our case, resistant plants are defined as those which develop no symptoms due to the virus and have background ELISA values during the entire experiment.

A large number of plants of lines SV 33, SV 98, SV 108 and SV 134 in both and SV 117 in the first resistance test had initially high OD_{405}, which decreased at 8 wpi and even in some cases reached background levels of ELISA (see Table 8 and Appendix). Kawchuck et al. (1991) found a similar phenomenon when challenging CP PLRV transformed potatoes with this virus, OD_{405} readings increased until 42 days after infection (dpi), and decreased when measured at 56 dpi. Contrarily all infected plants of the controls in our study reached high OD_{405} levels at the end of the experiment.

A large variability in the data of ELISA could be found in the transgenic lines tested, especially in those which had a weak protection against the virus. In some 5'3'S (SV 33) and ORF1/2 (SV 98 and SV 108) transgenic lines a few inoculated plants developed symptoms and were highly infected with virus as assessed by ELISA,

therefore they behaved similar to the inoculated controls. When only the inoculated plants of these lines which had low ELISA values at 8 wpi were grouped and their data were analysed an evident reduction in virus titer compared to the controls was found. This reduction in some cases was drastic (25 times for line SV 33 in the first resistance test), while in others it was not so strong but constant in both resistance tests (i.e. a 10 reduction in line SV 108). Although average ELISA values from plants with low final OD_{405} were 4 to 25 times lower than those found in the infected controls, it can not be concluded that plants were resistant to viral infection.

Some plants of line SV 33 had background levels during the entire experiment. In the first greenhouse test 5 plants showed this behaviour, while in the second resistance test 6 plants had low ELISA during the 8 weeks of the assay. Since all other inoculated plants of controls and transgenic lines were positive by ELISA in the first resistance test, it is unlikely that these 5 plants had escaped infection. However, in the second greenhouse resistance test some plants of a few transgenic escaped infection and this was related to the position of the plants in the greenhouse. According to the location of line SV 33 it was assumed that ca. 6 plants should be escapes, although it is difficult to assure that the low rate of infection was correlated with the position of plants in all lines tested.

Some virus challenged plants from lines SV 33, SV 98 and SV 108 had final height and weight similar to the respective uninoculated control. It is remarkable that line SV 33 had a large number of inoculated plants that were similar in height and weight as the healthy controls. The same was also found in virus infected plants of line SV 108, but to a lesser extent. These results agree with the low degree of protection against BWYV found in both lines as assessed by ELISA at 8 wpi.

Despite the results of ELISA, final height and weight of lines SV 33, SV 98 and SV 108, in no case 100% of the inoculated plants showed the same degree of protection. In all cases it was found that some plants were as susceptible to the virus as the controls.

The lines mentioned above had a similar response when challenged with BWYV in both resistance tests performed. The same is not true for lines SV 134 and SV 117, where although some inoculated plants reached low ELISA values and similar height and weight as the controls at the end of the experiment the results were not reproducible in both resistance tests. On one hand, in the second, but not in the first greenhouse resistance test, a large number of plants of line SV 134 which had a stronger response to virus than the controls were found (see Table 10). As mentioned before the rate of infection of the transgenic lines was low in the second assay, it can be possible that these plants were inoculated with a lesser amount of virus, therefore they could tolerate viral infection. On the other hand, an extremely opposite response to BWYV was observed in line SV 117 in both resistance tests. At

the end of the first experiment infected plants seemed not to have been greatly affected by the virus (see Table 5A) reaching in most cases low ELISA and normal height. However during the second resistance test 90% of the plants assayed were susceptible to virus inoculation, reaching ELISA values similar to the ones obtained in the inoculated controls. Barker et al. (1998) have observed that it is possible that the effectiveness of the transgene is partly determined by environmental conditions, since more transgenic plants were resistant in tests conducted in warmer weather than in those made in winter. In part this could explain the higher susceptibility of line SV 117 to virus inoculation in the second than in the first resistance test.

Audy et al. (1994) reported for the first time segregation for resistance in R1 and in R2 progeny of plants transformed with PVY replicase genes. Similar results were described by Brederode et al. (1995), where they assumed that in AlMV replicase transformed tobacco lines showed partial resistance, i.e. some plants of the same line were resistant and others completely susceptible to AlMV inoculation. Similar segregation could be occurring in some of the ORF1/2 lines, which also encode the replicase gene of BWYV or in 5´3´S plants tested in this study. Some of the T3 from these plants should be assayed for resistance against this virus and compare them to seedlings from plants which do not show this response. At the same time the analysis of individual plants of each of these lines for a possible correlation between protection and accumulation of the transcript could be done.

Up to date resistance found with other luteoviruses, mainly with PLRV, is based on the reduction of ELISA values. van der Wilk et al. (1991) observed that the CP of PLRV protects potatoes from infection with the virus. This study showed that the average ELISA values from some of the transgenic lines were diminished when comparing them to the infected controls (4 to 10 times). The number of plants tested was relatively low and at the same time no vector transformed line was included as control. In another study done by Presting et al. (1995) using CP PLRV transgenic potatoes, they observed that the vector controls challenged with the virus showed as well lower viral titers as assayed by ELISA than the untransformed plants. Therefore a somaclonal variation could have taken place. The authors postulated that the interaction between a phloem-limited virus and its host can be disrupted by a number of slight modifications to the plant growth pattern or physiology. Somaclonal variation was described in potatoes which were induced by regeneration via a callus phase (Potter and Jones, 1991). Kawchuck et al. (1991) used sense and antisense constructs of the CP from PLRV, obtaining high levels of resistance in some lines containing either of the constructs. No CP protein was detected, therefore the mechanism for resistance in this case could be RNA mediated. Tacke et al. (1996) transformed potatoes with the MP of PLRV and obtained a broad spectrum resistance against virus infection, once again the resistance was measured as decrease in ELISA values. Resistance against BWYV has been tested in lettuce using the CP either in sense or antisense orientation (Gielen et al., 1994). In all lines

tested no differences in ELISA values between the transgenic lines and the inoculated controls were found, therefore these constructs did not confer resistance against the virus.

It has been postulated that due to the phloem specificity of the luteoviruses, it may be difficult to obtain resistance using the common plant promoters (de Hann, 1998). Graham et al. (1997) have used two different phloem specific promoters, RolC and Sh (derived from maize) to transform potatoes with the CP from PLRV. The results of challenging the transgenic plants with virus showed that the average ELISA value from RolC-CP transformed plants was decreased compared to the controls, although large variation in OD_{405} was obtained. As with the study performed by van der Wilk et al. (1991) no vector transformed control was included.

Tenllado et al. (1995) have shown a recovery phenomenon in infected transgenic plants using *N. benthamiana* transformed with a truncated form of the PMMV replicase. In this case infected plants initially showed the same viral symptoms as the infected susceptible controls, but after some weeks the new emerging leaves showed no symptoms and were free of virus as determined by ELISA. The recovered plants had decreased final height (ca. 70% to 80%) compared to the healthy controls. A similar phenomenon was observed by Jones et al. (1998) in transgenic peas expressing the PSbMV replicase gene, but in this case the recovery phenomenon was associated with the absence of viral RNA and a dramatic reduction in the transgene RNA in the recovered leaves. In our study, although we observe reduction in ELISA with time and levels of decreased height similar to those described by Tenllado we can not assume that infected plants of a few lines showed this “recovery” phenomenon. First, BWYV inoculated plants, which initially had high OD_{405} levels did not show any delay in symptoms compared to the controls and secondly, the newly emerging leaves had low or in a few cases near to background levels of BWYV as assessed by ELISA, but not in all cases were completely virus-free.

4.2.4. MECHANISM OF RESISTANCE

The failure to obtain resistant *N. benthamiana* plants transformed with ORF1/2, 5'3'S and 5'3'AS of BWYV in this study may be ascribed to the fact that a truncated or mutated form of the polymerase may be required (Donson et al., 1993). On the other hand, some lines showed a low degree of protection against the virus compared to the controls.

If infected plants from line SV 33 are seen as "protected" against viral infection, then probably the mechanism underlying this resistance is RNA mediated, since no protein is expected to be synthesized by the 5'3'S viral fragment. The results of Northern blot show that this line expresses high levels of the transgene.

Two of the 5 ORF1/2 lines tested had a stronger response to BWYV than the controls in both resistance tests (SV 108 and SV 98). According to the results obtained by Northern blot line SV 108 had a very low expression of the transgene and as determined by Km segregation it had more than three copies of the transgene. In this case cosuppression could be acting as response mechanism against the virus. But the same is not true for line SV 98, where the transgene could be detected, although it also seemed to have more than 3 copies as seen by the results of Km segregation.

The detection of the viral protein was not possible in this study due to the lack of an antibody, therefore the probability of protein mediated protection can not be ruled out. However in most cases of RMR the viral polymerase has not been detected, since it is postulated that it can be synthesized in very low amounts, or can have a high turnover in the plant cell (Golemboski et al., 1990).

If the response of the plants tested in our study was due to gene silencing it should be expected that plants having multiple copies of the transgene (i.e. SV 108) would be involved in this response. In a few cases it has been observed that gene silencing occurs even when one insert is present in the plant genome (reviewed by Stam et al., 1997; Matzke and Matzke, 1998). In most cases it is postulated, that resistance should be associated with the ability to synthesize aberrant RNA (aRNA) rather than the amount of RNA expressed in the plant cells (Russo et al., 1998).

In summary it is difficult to compare our results with those obtained by others. Firstly, most of the resistant transgenic plants obtained up to date contain viral sequences from non phloemic specific RNA viruses. In a few cases sequences from DNA viruses, which are phloem specific (i.e. geminiviruses) have been used to transform plants and tested for resistance. Of the luteoviruses only PLRV has been tested for resistance in potatoes, although no immunity has been obtained, lower viral replication can be found as assessed by ELISA. Secondly, most greenhouse resistance tests have been performed with mechanical inoculation of the virus, only

in a few studies vectors carrying the virus have been used. Finally, in some cases no vector-transformed line has been included as susceptible control, so it can be questioned if the resistance obtained is due to the viral insert or to a somaclonal variation.

4.3. FUTURE STUDIES

It must be emphasised that only a small proportion of the total transgenic plants generated were tested for resistance in this study. Therefore it could be interesting to analyse the behaviour of the remaining lines, especially those containing either the ORF1/2 or the 5'3'S viral fragment, since they showed a stronger response to virus inoculation than the controls. In general when testing for resistance large numbers of transgenic lines must be analysed before ruling out the possibility that resistance is not conferred by a particular construct (Palukaitis and Zaitlin, 1997). As an example, Lomonossoff, (1995) found resistant plants carrying the 54-kDa sequence of PEBV, while MacFarlane and Davies, (1992) found no resistance in transgenic plants expressing truncated versions of PEBV 54-kDa sequences which may have been a consequence of examining few lines.

Besides it should be interesting to determine if this weak protection observed in some plants of the most interesting lines (SV 33, SV 98, SV 108, SV 117 and SV 134) is rather due to artificial factors, such as initial virus inoculum or to a better physiological condition of the plant when inoculated as described by Presting et al. (1995) or to resistance against BWYV.

Some points must be considered in future greenhouse resistance tests:

a) Use a larger number of aphids to transmit the virus and allow them to feed for longer periods.
b) Increase the amount of light during the AAP of the aphids.
c) Random position of the plants in the greenhouse.

Considering all these factors a new resistance test will be carried out with lines SV 33 (5'3'S), SV 98 and SV 108 (ORF1/2), which showed a weak protection against the virus and had a similar response in both greenhouse resistance tests. Of especial interest is line SV 33, since in both resistance test ca. 5 plants which had background ELISA levels during the 8 weeks of the experiment were found.

It is interesting that most of the RMR studies which have succeeded in conferring resistance against a determined pathogen, have used a truncated rather than the entire form of the viral replicase (Audy et al. 1994; Tenllado, 1995; Guo and Garcia, 1997; Anderson et al., 1992; Longstaff et al., 1993). Huet et al. (1999) have proposed

that in the case of Rep protein-mediated resistance for some viruses, an active Rep has to be expressed (i.e. PVY, Audy et al.; 1994.) and for some others, the Rep has to be defective (i.e. AlMV, Brederode et al.; 1992). Therefore, it could be interesting to use either a truncated or mutated form of the polymerase of BWYV to test for resistance against BWYV.

A phloem-specific promoter derived from coconut foliar decay virus (CFDV), which is a phloem specific virus, has been employed in the study of movement proteins of different viruses (Hehn and Rohde, 1998). The three constructs which were used in this study have been cloned into a new vector under the control of the CFDV promoter. The transformation of *N. benthamiana* plants with these constructs and further resistance tests could give some positive results for viral resistance.

Summary

N. benthamiana plants were transformed by means of *A. tumefaciens* with either the viral replicase (ORF1/2), one of two smaller sequences involving the 5‘ and 3‘ ends (5‘3‘S and 5‘3‘AS) of the genome of BWYV or with the pBin19 vector alone (Kp). In total ca. 120 kanamycin (Km) resistant plantlets were generated. Of these 115 were positive by NPTII ELISA and/or PCR for the transgene (50, 24, 25 and 16 plants containing the ORF1/2, 5‘3‘S, 5‘3‘AS and Kp, respectively). Plants were transferred to the greenhouse and allowed to self-pollinate. Seeds from the primary transformants (To) were collected and tested for segregation on different Km concentrations (100 to 300 mg/l). Seeds from ca. 30% of the lines tested did not germinate or had a poor growth at these antibiotic concentrations.

According to different criteria such as levels of NPTII ELISA of To lines, PCR, growth at 200 mg/l of Km and phenotype some lines were choosen as candidates to be tested for resistance against BWYV. 5 lines of each construct were randomly selected. These lines were analysed by Northern blot for expression of the transgene and/or the nptII gene. Different levels of expression of the transgene were found.

Greenhouse resistance tests were performed twice (from June to August 1999 and August to October 1999). 15 transgenic *N. benthamiana* lines (5 per construct) as well as two controls (vector transformed and untransformed plants) were tested. Km resistant plantlets were transferred to the greenhouse and 20 plants from each line were inoculated with BWYV by means of green peach aphids (*Myzus persicae*), while other 20 were kept as uninoculated control. At 4, 6 and 8 weeks post infection (wpi) an BWYV ELISA of the inoculated plants was carried out and the height of each plant was measured, while the weight was determined at the end of the experiment (8 wpi).

All 5‘3‘AS lines tested were susceptible to BWYV. Although plants from line SV 77 had low ELISA values at all times tested their final weight and height were similar as the infected controls.

Two 5‘3‘S transgenic lines (SV 33 and SV 134) were slightly protected against BWYV. Inoculated plants of line SV 33 had lower ELISA values at 8 wpi than at 4 wpi and as the rest of the lines tested. The final height and weight of these plants was ca. 80% of the respective uninoculated plants. A large number of “protected“ plants of line SV 134 were found mainly in the second resistance test.

Three ORF1/2 lines (SV 98, SV 108 and SV 117) seemed to have a weak protection against the virus. A large number of inoculated plants of lines SV 98 and SV 108 reached similar final weight and height as the uninoculated control and had low ELISA values at 8 wpi. Line SV 117 had a weak protection in the first greenhouse resistance test, while in the second test ca. 90% of the plants were susceptible to BWYV. It is possible that the different response observed in this line was due to environmental conditions. No correlation was found between levels of expression of the transgene and degree of protection. Only for line SV 108 an inverse correlation was found.

In summary all transgenic *N. benthamiana* lines tested were susceptible to BWYV. Despite these results 3 lines seemed to have a weak protection against the virus (SV 33, SV 98 and SV 108), therefore it should be interesting to further test them.

Zusammenfassung

N. bethamiana Pflanzen wurden mittels *Agrobakterien* mit der viralen Replikase (ORF1/2), einer fusionierte Sequenz, die das 5‘ und 3‘ Ende (5‘3‘S und 5‘3‘AS) des BWYV enthält, oder nur mit dem pBin19 Vektor (Kp) transformiert. Insgesamt wurden 120 kanamycin (Km)-resistente Pflanzen regeneriert. Davon waren 115 positiv im NPTII ELISA und/oder in der PCR (50, 24, 25 und 16 Pflanzen enthielten ORF1/2, 5‘3‘S, 5‘3‘AS bzw. Kp). Die Pflanzen wurden in das Gewächshaus überführt und geselbstet. Die Samen der Primärtransformanten (To) wurden durch zum Testen der Aufspaltung auf unterschiedlichen Km-Konzentrationen (100-300 mg/l) gehalten. Samen von ca. 30% der getesteten Linien keimten nicht oder zeigten bei dieser Antibiotika-Konzentration ein spärliches Wachstum.

Infolge unterschiedlicher Kriterien wie NPTII ELISA der To Linien, PCR, Wachstum auf 200 mg/l Km und Phänotyp wurden je 5 Linien pro Konstrukt als Kandidaten für den Resistenztest gegen BWYV ausgewählt. Diese Linien wurden im Northern blot auf die Expression der viralen Gensequenzen analysiert. Unterschiedliche Expressionsniveaus dieser Gene wurden gefunden.

Gewächshaus-Resistenztests wurden zweimal (von Juni bis August 1999 und August bis Oktober 1999) durchgeführt. 15 transgene Linien (5 je Konstrukt) und jeweils 2 Kontrollen (mit dem Vektor-transformierten und nicht transformierte Pflanzen) wurden getestet. Km-resistente Pflanzen wurden in das Gewächshaus überführt und 20 Pflanzen jeder Linie mittels Grüner Pfirsichblattlaus (*M. persicae*) mit BWYV inokuliert, während andere 20 nicht inokulierte Pflanzen als Kontrolle dienten. Jeweils 4, 6 und 8 Wochen nach Infektion erfolgten ein BWYV ELISA-Test der inokulierten Pflanzen, das Vermessen der Wuchshöhe und am Ende des Experiments (8 Wochen nach Infektion) das Bestimmen des Gewichts.

Alle getesteten 5‘3‘AS Linien waren anfällig gegen BWYV. Obwohl die Pflanzen der Linie SV 77 bei allen Tests einen niedrigen ELISA-Wert hatten, waren das Gewicht und die Höhe ähnlich denen der infizierten Kontrollen.

Zwei 5‘3‘S transgene Linien (SV 33 und SV 134) waren weniger anfällig gegen BWYV. Inokulierte Pflanzen der Linie SV 33 hatten 8 Wochen nach Infektion einen niedrigeren ELISA-Wert als 4 Wochen nach Infektion und als der Rest der getesteten Linien. Die Höhe und das Gewicht dieser Pflanzen entsprachen ca. 80% der Höhe und des Gewichtes der nicht inokulierten Pflanzen. Ein große Anzahl der weniger anfälligen Pflanzen der Linie SV 134 wurde vorwiegend im zweiten Resistenztest gefunden.

Drei ORF1/2 Linien (SV 98, SV 108 und SV 117) wiesen einen geringe Anfälligkeit gegen das Virus auf. Eine große Zahl der inokulierten Pflanzen der Linien SV 98 und

SV 108 erreichte ähnliches Endgewicht und eine ähnliche Endhöhe wie die nicht inokulierte Kontrolle und hatte 8 Wochen nach Infektion einen niedrigen ELISA-Wert. Linie SV 117 hatte im ersten Gewächshaus-Resistenztest eine verminderte Anfälligkeit, während im zweiten Test ca. 90% der Pflanzen anfällig gegenüber dem BWYV waren. Es ist möglich, dass die beobachtete unterschiedliche Reaktion in dieser Linie auf Umweltbedingungen zurückzuführen ist. Keine Korrelation wurde zwischen der Expressionshöhe der transgenen Sequenzen und dem Grad der geringeren Anfälligkeit gefunden. Nur für Linie SV 108 wurde eine negative Korrelation festgestellt.

Zusammengefasst waren alle getesteten transgenen *N. benthamiana*-Linien anfällig gegenüber BWYV. Ungeachtet dieser Resultate schienen drei Linien einen verminderte Anfälligkeit gegen das Virus zu haben (SV 33, SV 98 und SV 108) und es sollte interessant sein, sie weiterhin zu testen.

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7. Appendix

1. First greenhouse resistance test

2. Second greenhouse resistance test

In all Tables shown the abbreviations employed are:

ELISA I	BWYV ELISA at 4 wpi
ELISA II	BWYV ELISA at 6 wpi
ELISA III	BWYV ELISA at 8 wpi
h1i	Height from BWYV infected plant at 4 wpi
h2i	Height from BWYV infected plant at 6 wpi
h3i	Height from BWYV infected plant at 8 wpi
w3i	Weight from BWYV infected plant at 8 wpi
h1	Height from uninoculated plant at 4 wpi
h2	Height from uninoculated plant at 6 wpi
h3	Height from uninoculated plant at 8 wpi
w3c	Weight from uninoculated plant at 8 wpi

In all lines the average and the SD are shown at the bottom (in bold) for each case.

ORF1/2 First grennhouse resistance test

Line	N°	ELISAI	ELISA II	ELISA III	h1i	h2i	h3i	w3i	N°	h1c	h2c	h3c	w3c
98	1	0.887	3.697	2.920	17.0	27.5	34.0	4.7	1	33.0	49.5	58.0	29.9
	2	1.476	0.029	0.065	19.0	33.0	42.0	8.7	2	26.0	46.0	65.0	19.2
	3	0.936	0.188	0.650	23.0	29.5	30.0	10.8	3	32.0	51.0	62.0	24.8
	4	1.188	0.684	0.088	14.0	38.0	46.0	15.0	4	26.0	44.0	57.0	17.8
	5	0.187	0.112	0.000	27.0	44.0	56.0	27.6	5	28.0	44.0	55.0	26.3
	6	2.943	3.533	ns	15.0	18.0	21.0	0.3	6	27.0	47.5	63.0	21.3
	7	0.143	0.247	0.986	16.0	20.0	23.0	6.6	7	31.0	51.5	70.0	27.5
	8	0.547	0.223	0.000	17.0	36.0	47.0	18.7	8	24.5	46.0	60.0	18.3
	9	1.103	0.011	0.587	19.0	37.0	47.0	14.8	9	29.0	50.5	67.0	24.0
	10	2.749	0.054	0.029	13.0	29.5	41.0	11.5	10	31.0	53.5	67.0	26.9
	11	0.411	0.105	0.052	15.0	34.0	42.0	22.4	11	23.0	46.0	65.0	24.0
	12	1.687	2.309	0.000	23.0	40.0	48.0	14.7	12	31.0	57.0	70.0	20.5
	13	0.426	1.895	2.906	16.0	29.0	37.0	12.9	13	26.0	48.5	64.0	18.6
	14	1.234	0.277	0.366	20.0	41.0	50.0	16.9	14	23.0	37.0	44.0	16.2
	15	0.587	0.808	0.121	17.0	35.0	46.0	15.0	15	25.0	46.0	60.0	18.0
	16	1.846	0.333	0.133	23.0	29.5	36.0	11.5	16	27.0	49.0	66.0	26.3
	17	3.183	0.538	1.325	20.0	33.0	40.0	16.8	17	23.0	43.0	55.0	23.2
	18	2.579	1.075	0.013	16.0	30.5	39.0	15.7	18	30.0	53.5	67.0	21.3
	19	0.607	1.589	0.404	20.0	35.0	42.0	21.6	19	26.0	44.0	59.0	22.1
	20	0.311	0.150	0.000	24.0	48.0	59.0	32.0	20	24.0	42.0	55.0	19.3
		1.252	**0.893**	**0.560**	**18.7**	**33.4**	**41.3**	**14.9**		**27.3**	**47.5**	**61.5**	**22.3**
		0.770	**0.874**	**0.633**	**3.1**	**5.4**	**7.1**	**5.3**		**3.1**	**4.6**	**6.2**	**3.7**

Line	N°	ELISA I	ELISA II	ELISA III	h1i	h2i	h3i	w3i	N°	h1c	h2c	h3c	w3c
108	1	1.148	1.045	0.990	19.0	32.0	35.0	4.7	1	32.0	51.0	60.0	21.1
	2	0.294	2.147	ns	21.0	30.0	30.0	1.3	2	24.0	40.5	51.0	25.5
	3	1.042	1.124	0.495	28.0	48.0	60.0	23.4	3	27.0	50.5	63.0	22.2
	4	1.023	1.124	0.311	16.0	33.0	46.0	11.2	4	30.0	52.0	66.0	26.9
	5	0.396	0.103	1.355	16.0	35.0	45.0	13.4	5	29.0	52.0	65.0	20.3
	6	0.332	1.041	0.889	15.0	29.0	37.0	7.9	6	30.0	51.0	66.0	23.2
	7	1.032	1.241	1.376	21.0	36.0	42.0	8.3	7	28.0	47.5	63.0	27.6
	8	0.477	0.644	0.079	18.0	32.5	38.0	7.5	8	28.0	49.0	63.0	24.2
	9	1.266	1.045	0.055	23.0	44.5	55.0	22.6	9	26.0	48.0	61.0	24.9
	10	1.436	1.845	2.398	16.5	34.0	46.0	10.6	10	27.0	48.5	63.0	20.4
	11	0.838	0.784	0.070	23.0	40.0	52.0	27.6	11	26.0	45.0	60.0	20.6
	12	0.449	0.557	0.706	17.0	28.0	36.0	7.0	12	34.0	55.5	69.0	17.9
	13	1.369	0.894	0.037	26.0	44.0	54.0	24.6	13	32.0	54.0	68.0	16.5
	14	0.250	0.479	1.489	12.0	23.0	30.0	6.2	14	29.0	50.5	63.0	24.7
	15	0.359	1.121	0.531	13.5	26.0	40.0	11.6	15	27.0	52.0	65.0	20.9
	16	1.436	2.457	ns	18.0	31.0	32.0	2.2	16	23.0	39.0	57.0	28.0
	17	1.000	1.241	0.257	18.5	28.5	42.0	18.6	17	31.0	50.5	64.0	25.0
	18	0.227	0.114	0.007	22.0	39.5	51.0	28.7	18	31.0	49.0	59.0	21.1
	19	0.100	0.412	0.195	21.0	41.5	50.0	25.0	19	29.0	49.5	62.0	16.8
	20	0.095	0.864	0.264	14.5	27.0	31.0	6.3	20	23.0	42.0	60.0	28.8
		0.728	**1.014**	**0.639**	**18.9**	**34.1**	**42.6**	**13.4**		**28.3**	**48.9**	**62.4**	**22.8**
		0.431	**0.428**	**0.525**	**3.3**	**5.6**	**7.6**	**7.6**		**2.9**	**4.2**	**3.9**	**3.6**

ORF1/2 First greenhouse resistance test

Line	N°	ELISA I	ELISA II	ELISA III	h1i	h2i	h3i	w3i	N°	h1c	h2c	h3c	w3c
110	1	0.245	0.224	0.948	4.0	14.0	19.0	2.8	1	10.0	26.0	33.0	12.9
	2	0.503	2.056	0.309	5.0	14.0	22.5	4.3	2	14.5	29.0	40.0	28.6
	3	0.098	1.341	0.278	6.0	16.0	27.0	7.5	3	4.0	22.0	42.0	20.6
	4	0.259	0.294	0.945	6.0	15.0	20.0	3.6	4	8.0	31.5	47.0	20.5
	0	0.214	1.121	2.354	4.0	13.0	21.0	6.1	5	12.0	39.0	56.0	22.7
	6	0.495	0.069	1.124	6.0	20.0	32.0	6.7	6	7.0	25.0	40.0	19.7
	7	0.881	0.986	ns	4.5	7.0	7.0	0.2	7	13.0	31.5	43.0	16.1
	8	0.659	0.709	0.542	7.0	22.0	31.0	6.8	8	6.0	19.0	25.0	11.8
	9	0.571	0.072	1.874	2.0	7.0	8.0	0.1	9	8.0	19.5	28.0	8.9
	10	0.322	0.359	1.966	5.0	13.0	17.0	2.7	10	9.0	32.5	49.0	12.1
	11	1.138	0.811	1.478	2.0	5.0	7.0	1.0	11	14.0	34.5	52.0	12.5
	12	0.351	1.335	1.478	6.0	12.0	20.0	5.7	12	13.0	30.0	39.0	23.3
	13	1.269	0.525	3.479	5.0	11.0	15.0	2.0	13	11.0	28.0	40.0	27.2
	14	0.225	0.856	0.729	5.0	13.0	22.0	5.1	14	9.0	28.0	37.0	12.9
	15	1.509	0.749	1.315	4.0	16.5	22.0	4.6	15	10.0	34.5	49.0	14.8
	16	1.439	0.480	1.812	7.0	10.5	11.0	0.7	16	10.0	27.5	40.0	11.6
	17	0.631	1.721	2.095	5.0	13.0	19.0	3.4	17	12.0	35.5	53.0	22.2
	18	0.438	0.108	2.080	4.0	10.0	11.0	0.3	18	15.0	41.0	53.0	29.0
	19	1.023	1.916	1.986	4.5	10.0	17.0	4.9	19	9.0	28.0	46.0	23.3
	20	0.253	0.861	1.257	4.0	10.0	19.0	3.5	20	13.0	31.0	50.0	22.3
		0.626	**0.830**	**1.476**	**4.8**	**12.6**	**18.4**	**3.6**		**10.4**	**29.6**	**43.1**	**18.7**
		0.354	**0.473**	**0.615**	**1.0**	**3.1**	**5.4**	**1.9**		**2.9**	**5.6**	**8.2**	**6.1**

Line	N°	ELISA I	ELISA II	ELISA III	h1i	h2i	h3i	w3i	N°	h1c	h2c	h3c	w3c
112	1	0.164	0.252	2.354	3.0	8.0	12.0	0.7	1	17.0	26.0	47.0	24.9
	2	0.817	1.521	3.105	4.0	7.5	10.0	0.7	2	13.0	32.5	43.0	15.6
	3	0.661	0.573	1.145	6.0	16.0	22.0	3.2	3	14.0	39.5	53.0	31.2
	4	0.548	0.729	1.379	2.0	6.0	6.0	0.9	4	12.0	30.0	39.0	15.6
	5	0.000	0.076	0.874	9.0	19.0	24.0	18.1	5	14.0	35.0	55.0	29.5
	6	0.348	0.850	3.112	2.0	5.5	6.0	2.6	6	11.0	28.0	46.0	30.1
	7	0.285	1.872	3.232	4.5	12.5	18.0	1.6	7	20.0	45.0	61.0	26.5
	8	1.210	0.715	0.569	3.5	15.0	23.5	2.9	8	19.0	35.5	47.0	20.6
	9	0.547	0.526	0.678	8.0	23.0	33.0	7.5	9	11.0	30.5	44.0	16.3
	10	0.000	0.031	0.654	4.0	20.5	31.0	7.1	10	3.0	8.5	16.0	21.0
	11	0.113	0.563	1.538	5.0	14.5	18.0	2.0	11	14.0	30.0	46.0	13.6
	12	0.108	0.133	1.780	2.0	9.0	12.0	1.0	12	12.0	25.5	36.0	9.3
	13	0.342	2.419	0.845	7.5	21.0	25.0	3.6	13	15.0	35.0	49.0	24.3
	14	0.471	1.092	2.312	4.0	10.0	14.0	2.2	14	9.0	20.5	30.0	13.4
	15	0.458	0.973	2.382	1.0	7.0	12.0	6.4	15	12.0	32.0	44.0	14.1
	16	0.319	1.597	0.728	5.0	13.0	20.0	6.9	16	12.0	36.0	51.0	22.0
	17	0.383	1.337	2.002	3.5	11.0	15.5	3.3	17	21.0	40.0	55.0	26.9
	18	0.294	0.254	ns	5.0	14.0	14.0	0.1	18	17.0	35.0	53.0	20.2
	19	0.403	0.824	1.031	4.0	13.0	20.0	2.1	19	23.0	44.0	57.0	31.0
	20	0.555	0.137	2.881	5.0	16.0	20.0	1.2	20	24.0	44.0	56.0	28.0
		0.401	**0.824**	**1.716**	**4.4**	**13.1**	**17.8**	**3.7**		**14.7**	**32.6**	**46.4**	**21.7**
		0.206	**0.507**	**0.812**	**1.5**	**4.1**	**5.9**	**2.7**		**4.9**	**8.4**	**10.2**	**6.6**

ORF1/2 First greenhouse resistance test

Line	N°	ELISA I	ELISA II	ELISA III	h1i	h2i	h3i	w3i	N°	h1c	h2c	h3c	w3c
117	1	0.371	0.099	0.081	16.5	35.0	47.0	11.7	1	28.0	48.0	61.0	21.0
	2	0.979	0.000	0.084	19.0	40.0	52.0	15.2	2	26.0	47.0	59.0	24.6
	3	0.363	0.528	0.319	14.0	38.0	48.0	13.3	3	23.0	45.5	60.0	22.0
	4	0.257	0.141	0.000	20.0	41.0	53.0	16.9	4	19.0	39.0	53.0	19.0
	5	0.628	0.765	0.000	13.0	26.5	36.0	5.9	5	24.0	48.0	63.0	27.8
	6	0.481	0.032	0.300	19.0	39.0	46.0	7.7	6	25.0	45.0	64.0	23.5
	7	0.311	0.054	0.000	20.0	47.0	60.0	16.3	7	19.0	39.0	56.0	12.3
	8	1.026	0.220	0.000	22.0	40.0	52.0	21.3	8	24.0	44.0	58.0	18.5
	9	1.570	0.252	0.000	17.0	36.0	48.0	15.3	9	24.0	43.0	52.0	21.7
	10	0.378	0.147	0.043	18.0	37.0	46.0	19.0	10	29.0	47.0	67.0	18.5
	11	0.264	0.000	0.000	14.0	37.5	51.0	15.0	11	21.0	43.0	63.0	23.2
	12	0.327	0.233	0.000	17.0	33.0	45.0	16.1	12	25.0	47.0	60.0	17.5
	13	0.769	0.015	0.017	16.0	32.0	37.0	6.7	13	25.5	44.0	61.0	25.0
	14	0.613	0.226	1.996	14.0	30.0	36.0	6.6	14	19.0	33.0	49.0	15.3
	15	0.237	0.329	1.332	11.0	22.0	30.0	7.0	15	25.0	46.5	61.0	20.2
	16	0.717	0.030	0.658	16.0	33.5	46.0	14.0	16	27.0	47.0	65.0	23.7
	17	0.260	0.454	0.000	17.0	32.0	37.0	9.2	17	26.0	45.0	64.0	18.5
	18	0.357	0.127	0.102	18.0	38.5	45.0	2.7	18	22.0	43.0	59.0	16.2
	19	2.302	1.159	0.133	17.0	32.0	40.0	10.6	19	28.0	46.0	68.0	28.7
	20	2.194	0.109	0.426	7.0	21.0	27.0	3.9	20	18.0	37.5	54.0	25.5
		0.720	**0.246**	**0.275**	**16.3**	**34.6**	**44.1**	**11.7**		**23.9**	**43.9**	**59.8**	**21.1**
		0.452	**0.201**	**0.338**	**2.5**	**4.9**	**6.6**	**4.5**		**3.2**	**3.8**	**4.9**	**4.1**

Controls for ORF1/2 First greenhouse resistance test

Line	N°	ELISA I	ELISA II	ELISA III	h1i	h2i	h3i	w3i	N°	h1c	h2c	h3c	w3c
158	1	0.931	0.843	0.986	4.5	16.0	28.0	5.7	1	15.0	30.0	47.0	18.9
	2	0.875	2.721	2.784	5.0	15.5	21.0	3.8	2	15.0	31.5	52.0	22.7
	3	0.406	1.293	2.354	4.0	13.5	22.0	6.2	3	14.0	31.0	48.0	16.0
	4	0.581	1.745	2.844	2.5	15.0	23.0	3.9	4	23.0	39.5	43.0	14.0
	5	0.771	1.357	2.278	4.0	15.5	25.0	4.7	5	19.0	32.5	50.0	28.1
	6	0.647	1.151	2.718	5.0	16.0	25.5	4.1	6	14.0	32.0	48.0	26.1
	7	1.124	1.222	1.241	4.0	18.0	31.0	3.8	7	17.0	38.0	55.0	26.9
	8	1.396	1.512	1.635	4.0	17.0	27.0	1.7	8	18.0	32.0	38.0	23.0
	9	0.603	2.624	1.758	4.5	13.5	19.0	1.1	9	21.0	44.5	62.0	9.5
	10	1.498	1.543	3.453	2.0	9.5	15.0	2.2	10	24.0	42.5	56.0	22.7
	11	1.063	2.748	2.397	3.0	12.0	20.0	1.2	11	19.0	37.0	53.0	9.8
	12	0.911	1.446	2.573	2.0	8.0	11.0	4.9	12	15.0	34.0	49.0	15.6
	13	1.075	1.627	2.645	3.0	10.5	18.0	5.5	13	16.0	36.5	54.0	18.6
	14	0.569	1.162	2.156	4.0	10.0	17.0	7.9	14	15.0	34.5	55.0	16.8
	15	0.696	2.501	3.242	2.0	7.0	11.0	4.4	15	16.0	33.5	48.0	16.9
	16	1.441	1.558	2.906	4.0	10.5	16.5	2.5	16	15.0	29.5	42.0	14.0
	17	0.306	2.531	2.924	3.0	11.0	18.0	6.3	17	15.0	32.0	45.0	23.2
	18	0.531	2.583	3.608	3.0	9.5	16.0	3.6	18	12.0	31.5	46.0	12.4
	19	1.940	0.959	2.367	5.0	13.0	19.0	6.7	19	14.0	34.0	43.0	15.0
	20	1.505	2.381	3.620	5.0	13.0	21.0	1.7	20	15.0	33.0	49.0	26.5
		0.943	**1.775**	**2.524**	**3.7**	**12.7**	**20.2**	**4.1**		**16.6**	**34.4**	**49.1**	**18.8**
		0.349	**0.566**	**0.555**	**0.9**	**2.6**	**4.2**	**1.5**		**3.1**	**4.0**	**5.5**	**5.6**

Line	N°	ELISA I	ELISA II	ELISA III	h1i	h2i	h3i	w3i	N°	h1c	h2c	h3c	w3c
Nb	1	0.390	0.887	2.162	6.0	13.5	22.0	2.5	1	22.0	48.5	62.0	23.2
	2	2.270	2.120	1.987	6.5	17.0	20.0	3.5	2	10.0	42.0	60.0	24.6
	3	1.390	1.650	1.655	4.0	16.5	28.0	4.2	3	15.0	35.5	54.0	16.2
	4	0.978	1.144	1.587	8.0	15.0	24.0	8.6	4	16.0	39.5	55.0	20.1
	5	1.150	1.020	1.985	5.0	13.5	17.0	7.8	5	18.0	37.0	56.0	22.0
	6	1.353	1.144	2.302	7.0	11.0	16.5	1.0	6	20.0	39.0	61.0	20.3
	7	1.357	1.606	3.274	4.5	14.5	21.0	6.1	7	13.0	35.0	51.0	12.4
	8	2.097	2.450	2.622	4.0	13.5	21.0	3.0	8	14.0	40.5	56.0	15.5
	9	1.025	0.999	2.788	8.0	12.0	17.0	2.6	9	13.0	39.0	56.0	18.9
	10	2.675	1.822	2.624	9.0	17.0	25.0	4.6	10	16.0	42.5	61.0	21.3
	11	2.470	2.401	2.432	8.0	20.0	29.0	8.1	11	19.0	37.5	57.0	21.7
	12	2.257	1.985	1.722	5.5	21.0	31.0	4.9	12	18.0	38.5	58.0	17.6
	13	2.127	1.236	3.061	5.0	8.5	13.0	1.7	13	16.0	31.0	44.0	9.8
	14	2.879	2.066	1.854	8.0	8.0	8.0	0.3	14	16.0	36.0	52.0	11.5
	15	1.500	2.016	1.890	3.0	13.0	14.5	1.2	15	16.5	41.5	60.0	18.6
	16	0.987	1.789	2.145	5.0	13.0	20.0	2.2	16	19.0	45.0	64.0	25.6
	17	0.965	1.711	1.878	4.0	10.0	13.0	1.7	17	17.0	41.5	63.0	24.9
	18	1.452	1.038	1.554	9.0	17.0	21.0	10.0	18	16.0	37.0	54.0	18.4
	19	2.550	1.837	1.390	8.5	15.0	23.0	2.4	19	20.0	24.0	41.0	7.4
	20	1.655	1.498	1.043	5.0	13.0	20.0	1.6	20	23.0	46.0	51.0	36.3
		1.676	**1.621**	**2.098**	**6.2**	**14.1**	**20.2**	**3.9**		**16.9**	**38.8**	**55.8**	**19.3**
		0.591	**0.402**	**0.453**	**1.7**	**2.6**	**4.3**	**2.3**		**3.1**	**5.2**	**5.8**	**6.3**

5‘3‘S First greenhouse resistance test

Line	N°	ELISA I	ELISA II	ELISA III	h1i	h2i	h3i	w3i	N°	h 1	h 2	h 3	w3c
31	1	0.047	0.158	1.140	17.0	29.5	31.0	6.5	1	28.0	57.0	70.0	34.0
	2	0.673	2.057	3.599	12.0	23.0	31.0	4.3	2	29.0	59.0	71.0	26.5
	3	0.715	0.655	1.184	3.0	4.0	4.0	0.2	3	34.0	60.0	72.5	16.4
	4	0.335	2.705	2.902	16.0	25.0	29.0	2.6	4	34.0	55.0	66.0	14.2
	5	0.330	1.826	3.659	14.5	26.5	34.0	8.4	5	31.0	57.0	72.0	22.2
	6	0.136	1.230	2.476	16.0	30.0	36.0	7.7	6	31.5	63.0	78.0	36.0
	7	0.081	0.983	0.471	15.5	28.0	39.0	6.1	7	24.0	42.0	59.0	14.6
	8	0.220	1.277	0.683	20.0	34.0	42.0	7.4	8	32.0	40.0	66.0	23.5
	9	0.874	2.846	3.560	14.5	26.0	34.0	8.3	9	30.0	50.0	62.0	12.7
	10	0.106	1.309	3.137	17.0	26.0	30.0	6.4	10	26.0	44.0	54.0	11.3
	11	0.255	1.806	2.670	17.0	25.0	30.0	5.4	11	29.5	49.0	62.0	14.6
	12	0.730	1.218	2.382	16.5	29.5	39.5	11.3	12	26.0	49.0	63.0	12.5
	13	0.844	1.268	1.304	12.0	21.0	29.0	5.3	13	29.0	55.0	69.0	27.7
	14	0.303	1.880	2.600	13.5	23.0	28.5	3.7	14	31.0	54.0	67.0	22.7
	15	0.201	2.563	2.840	3.0	7.0	9.0	0.4	15	21.0	33.0	44.0	6.5
	16	0.630	1.573	2.992	16.0	24.0	28.0	2.4	16	29.0	52.0	67.0	17.3
	17	0.314	0.575	3.114	11.0	18.0	22.0	2.3	17	29.0	52.0	64.0	16.3
	18	0.019	1.112	1.371	16.0	28.0	39.0	10.0	18	28.0	51.0	66.0	24.7
	19	1.191	2.319	2.155	16.5	30.0	40.0	5.5	19	28.5	55.0	69.0	18.0
	20	0.054	1.313	1.784	25.0	33.0	38.0	4.0	20	23.0	40.0	50.0	22.3
		0.403	**1.534**	**2.301**	**14.6**	**24.5**	**30.7**	**5.4**		**28.7**	**50.8**	**64.6**	**19.7**
		0.284	**0.577**	**0.832**	**3.3**	**5.2**	**6.6**	**2.4**		**2.5**	**6.0**	**5.9**	**6.2**

Line	N°	ELISA I	ELISA II	ELISA III	h1i	h2i	h3i	w3i	N°	h1c	h2c	h3c	w3c
33	1	0.164	1.084	0.779	25.5	37.0	51.0	26.6	1	28.0	54.0	62.0	35.3
	2	0.462	1.032	0.068	25.5	39.0	44.0	11.5	2	31.0	61.0	73.0	27.5
	3	0.000	0.000	0.033	20.0	31.0	38.0	3.5	3	31.0	52.0	62.0	12.1
	4	0.000	0.000	0.036	31.0	57.5	63.0	15.3	4	30.5	53.0	66.0	12.9
	5	0.127	0.524	0.080	25.0	51.0	61.0	16.6	5	31.0	72.0	83.0	31.6
	6	0.123	0.304	0.013	26.0	53.0	65.0	32.6	6	35.0	61.0	74.0	34.3
	7	0.274	0.892	1.819	23.0	39.0	50.0	16.5	7	35.0	65.0	78.0	36.9
	8	0.274	0.722	0.242	20.0	38.5	44.0	7.4	8	32.5	59.0	76.0	25.1
	9	0.059	0.510	0.304	23.0	31.0	35.0	3.7	9	31.0	47.0	58.0	12.1
	10	0.000	0.000	0.000	32.0	54.0	61.0	11.8	10	26.5	38.0	51.0	10.9
	11	0.132	0.546	0.901	24.0	41.0	50.0	9.7	11	35.0	62.0	75.0	20.6
	12	0.170	0.474	0.017	27.0	56.5	67.0	30.8	12	36.0	65.0	78.0	27.1
	13	0.034	0.117	0.478	21.0	41.0	51.0	24.0	13	32.0	58.0	73.0	41.2
	14	0.678	0.713	2.153	22.0	37.0	43.0	9.8	14	33.5	52.0	63.0	12.7
	15	0.000	0.014	0.000	19.0	29.0	31.0	1.9	15	25.5	39.0	50.0	9.5
	16	0.487	0.531	0.046	16.5	26.5	29.0	2.7	16	29.0	42.0	49.0	13.1
	17	0.129	0.202	0.024	22.0	34.0	38.0	5.6	17	36.5	69.0	81.0	31.9
	18	0.772	0.345	0.000	25.0	50.0	63.0	24.2	18	32.5	65.0	80.0	36.9
	19	0.000	0.000	0.030	29.0	53.0	63.0	10.3	19	25.0	47.0	60.0	19.9
	20	0.478	0.950	0.390	17.5	32.0	44.0	18.9	20	22.5	44.0	57.0	19.5
		0.218	**0.448**	**0.371**	**23.7**	**41.6**	**49.6**	**14.2**		**30.9**	**55.3**	**67.4**	**23.6**
		0.190	**0.305**	**0.430**	**3.3**	**8.4**	**9.9**	**7.8**		**2.9**	**8.4**	**9.6**	**9.2**

5‘3‘S First greenhouse resistance test

Line	N°	ELISA I	ELISA II	ELISA III	h1i	h2i	h3i	w3i	N°	h1c	h2c	h3c	w3c
88	1	0.040	0.457	1.154	16.0	26.0	37.0	8.5	1	22.0	48.0	60.0	20.3
	2	0.033	0.340	1.767	9.5	19.0	25.0	2.7	2	15.0	33.0	41.0	5.9
	3	0.031	0.377	1.665	8.5	17.0	22.0	2.1	3	19.5	40.0	49.0	7.6
	4	0.278	0.359	0.855	6.0	10.0	15.0	0.6	4	23.0	42.0	52.0	10.5
	5	0.000	0.893	1.388	13.5	21.0	25.0	4.6	5	21.0	42.0	51.0	15.0
	6	0.023	0.430	0.996	6.5	14.0	16.0	1.7	6	20.0	50.0	64.0	40.4
	7	0.430	1.580	3.526	5.5	8.5	14.0	4.1	7	17.0	35.0	51.0	13.7
	8	0.099	1.664	3.602	15.0	22.5	26.0	3.6	8	13.0	25.0	34.0	8.8
	9	0.000	0.987	1.245	14.5	23.0	25.0	1.2	9	18.0	32.0	40.0	22.0
	10	0.000	0.815	1.368	11.5	19.0	22.0	3.0	10	15.0	22.0	37.0	10.0
	11	0.082	1.112	1.291	7.0	9.0	10.0	0.4	11	22.0	40.0	52.0	18.5
	12	0.303	0.784	1.744	7.0	10.0	16.0	1.6	12	23.0	41.0	51.0	10.7
	13	0.020	0.377	1.341	10.5	18.0	20.0	1.5	13	22.0	45.0	59.0	33.6
	14	0.025	0.112	1.011	7.5	12.0	13.0	0.8	14	14.0	27.0	37.0	7.2
	15	0.987	0.891	0.744	6.5	10.0	13.0	0.9	15	19.0	37.0	50.0	9.8
	16	0.514	1.624	3.744	3.5	5.0	6.0	0.6	16	21.0	42.0	60.0	21.7
	17	0.830	1.457	3.693	6.0	11.0	17.0	1.4	17	24.0	44.0	58.0	25.9
	18	0.008	0.636	1.119	7.0	15.0	27.0	5.3	18	16.0	38.0	50.0	26.8
	19	0.351	0.918	2.088	12.5	24.0	31.0	7.2	19	13.0	28.0	37.0	7.5
	20	0.000	1.258	2.066	16.0	30.0	40.0	9.7	20	15.5	32.0	41.0	7.8
		0.203	**0.854**	**1.820**	**9.5**	**16.2**	**21.0**	**3.1**		**18.6**	**37.2**	**48.7**	**16.2**
		0.227	**0.385**	**0.780**	**3.4**	**5.7**	**7.0**	**2.1**		**3.0**	**5.9**	**7.0**	**7.7**

Line	N°	ELISA I	ELISA II	ELISA III	h1i	h2i	h3i	w3i	N°	h1c	h2c	h3c	w3c
134	1	0.002	0.214	0.145	25.0	49.0	53.0	18.0	1	24.0	40.0	48.0	28.3
	2	0.067	3.323	1.726	19.0	24.0	33.0	3.7	2	28.0	50.0	61.0	16.3
	3	0.002	0.457	1.741	22.0	27.0	34.0	7.7	3	29.0	47.0	56.0	11.9
	4	0.252	1.050	1.838	16.5	21.0	26.0	2.3	4	29.0	50.0	55.0	12.4
	5	0.654	0.874	0.345	23.0	32.0	34.0	7.7	5	27.5	49.0	59.0	19.6
	6	0.121	0.478	0.442	18.0	29.0	34.0	7.7	6	27.5	59.0	65.0	33.0
	7	0.063	0.182	1.141	19.0	24.0	32.0	10.2	7	30.0	54.0	67.0	24.4
	8	0.784	0.698	0.458	23.0	27.0	31.0	7.0	8	32.0	50.0	57.0	20.4
	9	0.527	0.760	1.920	19.5	24.0	28.0	4.4	9	27.5	45.0	53.0	14.7
	10	0.069	0.483	2.266	19.0	26.0	28.0	2.7	10	34.5	56.0	65.0	16.7
	11	0.097	2.395	0.478	21.0	35.0	39.0	4.9	11	33.5	61.0	73.0	23.2
	12	0.784	0.984	0.387	19.0	22.0	27.0	6.9	12	32.0	57.0	68.0	32.8
	13	0.290	1.114	1.664	17.0	32.5	40.0	8.5	13	30.0	44.0	63.0	24.9
	14	1.861	3.093	1.468	15.0	24.0	26.0	4.2	14	33.0	60.0	73.0	20.7
	15	0.008	1.970	0.339	18.5	35.0	38.0	3.7	15	26.5	43.0	53.0	7.4
	16	0.692	2.579	1.834	17.0	28.0	32.0	4.1	16	28.5	50.0	63.0	17.9
	17	0.256	2.480	1.502	16.0	23.0	28.0	3.5	17	19.0	36.0	58.0	11.7
	18	0.108	1.650	2.066	15.0	31.5	40.0	11.2	18	29.0	51.0	62.0	31.6
	19	0.319	0.911	1.620	20.5	33.0	37.0	7.9	19	26.0	50.0	62.0	27.6
	20	0.432	1.438	0.382	18.0	24.0	31.0	8.4	20	21.0	39.0	49.0	25.1
		0.369	**1.357**	**1.143**	**19.1**	**28.6**	**33.6**	**6.7**		**28.4**	**49.6**	**60.5**	**21.**
		0.315	**0.807**	**0.707**	**2.1**	**4.9**	**4.7**	**2.7**		**2.8**	**5.3**	**5.6**	**6.2**

5‘3‘S First greenhouse resistance test

Line	N°	ELISA I	ELISA II	ELISA III	h1i	h2i	h3i	w3i	N°	h1c	h2c	h3c	w3c
135	1	0.129	0.467	0.647	18.0	30.0	36.0	9.3	1	24.0	52.0	61.0	32.9
	2	0.235	0.874	0.774	24.0	28.0	41.0	9.1	2	20.0	42.0	52.0	17.8
	3	0.084	0.817	1.118	14.0	20.0	22.0	3.1	3	24.0	42.0	52.0	15.3
	4	0.159	1.023	1.358	21.0	34.5	40.0	6.0	4	36.0	58.0	65.0	25.6
	5	0.354	0.457	0.945	20.0	27.0	33.0	8.4	5	33.5	60.0	71.0	32.1
	6	0.003	1.249	2.460	13.5	29.0	35.0	8.6	6	32.0	55.0	65.0	44.2
	7	0.660	2.245	3.659	17.5	30.0	36.0	8.3	7	25.0	52.0	65.0	24.2
	8	0.141	1.538	1.440	16.0	26.5	29.0	4.2	8	18.0	45.0	60.0	12.4
	9	0.987	1.540	1.471	23.5	36.5	39.0	6.1	9	25.0	43.0	54.0	9.5
	10	0.053	1.150	1.351	16.0	30.0	31.0	5.2	10	24.0	38.0	43.0	3.6
	11	0.050	1.384	3.903	15.5	29.5	35.0	4.5	11	22.5	37.0	41.0	9.9
	12	0.123	2.201	0.985	21.0	35.5	42.0	8.5	12	34.0	55.0	70.0	34.8
	13	0.170	1.832	3.397	14.5	24.0	27.0	4.1	13	18.0	40.0	62.0	18.8
	14	0.199	1.258	1.569	13.0	21.0	23.0	3.3	14	23.0	46.0	56.0	16.0
	15	0.260	2.824	3.663	10.0	14.0	14.0	1.0	15	17.0	30.0	37.0	6.5
	16	0.193	1.039	0.994	14.5	23.0	28.0	3.2	16	20.0	41.0	55.0	14.6
	17	0.499	1.181	1.303	17.0	27.0	34.0	7.9	17	25.5	49.0	62.0	19.7
	18	0.857	2.462	2.766	11.0	17.0	24.0	5.0	18	27.0	50.0	63.0	33.2
	19	0.078	2.905	2.641	18.0	34.0	38.0	3.5	19	17.5	40.0	57.0	39.9
	20	0.635	0.875	1.024	22.0	25.0	27.0	4.6	20	17.5	37.0	49.0	10.0
		0.293	**1.466**	**1.873**	**17.0**	**27.1**	**31.7**	**5.7**		**24.2**	**45.6**	**57.0**	**21.1**
		0.223	**0.582**	**0.938**	**3.2**	**4.6**	**6.0**	**2.1**		**4.5**	**6.7**	**7.4**	**9.8**

5‘3‘AS First greenhouse resistance test

Line	N°	ELISA I	ELISA II	ELISA III	h1i	h2i	h3i	w3i	N°	h1c	h2c	h3c	w3c
28	1	0.046	0.316	3.771	8.5	22.0	34.5	8.6	1	26.0	47.0	61.0	33.3
	2	0.015	0.216	2.286	12.0	23.5	33.5	3.8	2	29.0	52.0	64.0	19.0
	3	0.553	0.816	1.137	16.5	27.5	30.0	1.1	3	27.0	54.0	67.0	28.4
	4	0.430	1.352	0.594	18.0	34.0	44.0	1.1	4	29.0	58.0	63.0	30.2
	5	0.140	1.390	3.804	17.0	20.0	24.0	7.7	5	27.0	50.0	64.5	25.5
	6	0.127	0.203	0.644	12.0	20.0	26.0	3.1	6	15.0	36.0	53.0	16.7
	7	1.058	2.818	3.698	12.0	18.0	21.0	2.3	7	28.0	50.0	64.0	18.9
	8	0.224	2.086	3.866	10.0	17.0	21.0	2.5	8	30.0	49.0	62.0	24.8
	9	0.279	1.003	3.692	13.0	22.0	27.0	3.4	9	30.0	48.0	61.5	14.3
	10	0.192	1.022	3.844	9.0	16.5	24.0	2.7	10	24.5	37.0	49.0	8.5
	11	0.313	1.416	3.743	10.5	19.0	27.0	3.2	11	23.0	39.0	51.0	9.8
	12	0.209	1.845	3.670	13.0	23.0	31.0	6.0	12	29.5	55.0	65.0	30.4
	13	0.613	2.441	3.665	8.0	20.0	26.0	1.5	13	24.0	45.0	62.0	31.0
	14	0.105	0.590	3.647	13.5	23.5	32.5	4.5	14	21.0	37.0	44.0	15.9
	15	0.015	1.162	3.289	13.0	21.0	27.0	1.8	15	7.0	17.0	28.0	4.7
	16	0.835	3.155	3.717	8.0	15.0	19.0	1.5	16	11.0	25.0	34.0	8.5
	17	0.751	1.108	3.033	10.0	20.0	26.5	5.3	17	21.0	39.0	53.0	22.4
	18	1.231	2.631	1.695	11.0	22.0	32.0	7.5	18	26.0	59.0	72.0	33.5
	19	1.154	2.691	1.148	13.0	27.0	40.0	7.4	19	20.0	34.0	44.0	35.3
	20	0.819	1.794	3.471	12.5	22.0	28.0	4.3	20	23.0	48.0	60.0	25.6
		0.455	**1.503**	**2.921**	**12.0**	**21.6**	**28.7**	**4.0**		**23.5**	**43.9**	**56.1**	**21.8**
		0.337	**0.744**	**1.002**	**2.1**	**3.0**	**4.8**	**1.9**		**4.7**	**8.8**	**9.3**	**8.0**

Line	N°	ELISA I	ELISA II	ELISA III	h1i	h2i	h3i	w3i	N°	h1c	h2c	h3c	w3c
77	1	0.050	0.566	0.214	10.0	26.0	27.0	8.1	1	35.5	60.0	72.0	31.4
	2	0.098	0.277	0.179	16.0	22.0	25.0	7.2	2	32.5	41.0	68.0	17.4
	3	0.036	0.337	0.213	15.5	18.0	21.0	7.9	3	18.0	31.0	41.0	7.5
	4	0.025	0.400	0.166	13.0	21.0	24.0	2.9	4	29.5	56.0	68.0	24.6
	5	0.011	0.582	0.174	12.0	20.0	23.0	2.5	5	30.0	57.0	69.0	22.9
	6	0.040	0.112	1.571	16.0	19.0	22.0	8.1	6	26.0	54.0	63.0	40.2
	7	0.011	0.619	0.832	12.0	14.0	19.0	7.5	7	33.0	57.0	75.0	34.6
	8	0.905	2.052	1.642	13.0	18.0	21.0	7.7	8	10.0	22.0	30.0	5.6
	9	0.700	0.644	0.227	15.0	26.0	32.0	5.5	9	29.0	52.0	65.0	17.7
	10	0.047	0.200	1.144	18.0	24.0	28.0	8.8	10	27.5	47.0	60.0	17.6
	11	0.071	0.460	0.256	14.5	26.0	26.0	5.6	11	24.5	20.0	50.0	14.1
	12	0.015	0.283	0.289	16.0	20.0	24.0	4.8	12	25.5	51.0	66.0	29.6
	13	0.037	0.208	0.560	18.0	25.0	27.0	7.7	13	32.0	55.0	64.0	44.2
	14	1.400	1.007	1.048	2.5	6.5	7.0	0.4	14	28.0	52.0	64.0	26.6
	15	0.644	1.234	1.198	13.0	21.0	25.0	2.6	15	13.0	33.0	47.0	17.4
	16	0.172	0.616	0.819	7.0	12.0	15.0	0.8	16	21.5	35.0	40.0	11.1
	17	0.187	0.189	0.245	21.0	31.0	33.0	5.0	17	28.5	53.0	67.0	19.3
	18	0.108	0.237	0.195	17.5	23.0	31.0	6.9	18	24.5	47.0	59.0	26.2
	19	0.198	2.016	0.874	16.0	22.0	26.0	3.0	19	24.0	43.0	55.0	9.0
	20	0.047	0.254	0.145	21.0	24.0	27.0	4.4	20	25.5	52.0	63.0	23.1
		0.240	**0.615**	**0.600**	**14.4**	**20.9**	**24.2**	**5.4**		**25.9**	**45.9**	**59.3**	**22.0**
		0.269	**0.389**	**0.433**	**3.2**	**4.0**	**4.1**	**2.2**		**4.7**	**9.6**	**9.3**	**8.3**

5‘3‘AS First greenhouse resistance test

Line	N°	ELISA I	ELISA II	ELISA III	h1i	h2i	h3i	w3i	N°	h1c	h2c	h3c	w3c
86	1	0.585	1.738	0.945	16.0	27.0	29.0	8.8	1	20.0	40.0	54.0	22.1
	2	0.183	0.741	0.665	14.0	22.0	33.0	10.4	2	27.5	54.0	62.0	20.4
	3	0.147	2.357	3.557	12.5	25.0	32.0	5.4	3	30.0	60.0	68.0	22.7
	4	0.109	1.326	3.919	13.0	15.0	20.0	4.0	4	23.0	54.0	65.0	16.0
	5	0.046	0.449	3.439	11.0	26.0	33.0	8.1	5	26.5	52.0	62.0	17.4
	6	0.026	0.899	0.553	13.0	18.0	27.0	7.4	6	31.0	61.0	75.0	41.1
	7	0.047	2.598	0.618	15.0	25.0	32.0	9.2	7	28.0	51.0	46.0	16.3
	8	0.884	1.222	1.247	9.0	12.0	12.0	4.3	8	25.5	43.0	50.0	10.3
	9	1.012	2.170	3.767	18.0	20.0	22.0	4.6	9	29.0	50.0	60.0	11.3
	10	0.509	1.481	3.769	14.0	25.0	27.0	4.3	10	29.0	55.0	66.0	12.7
	11	0.000	0.547	0.974	14.0	25.0	29.0	5.0	11	31.0	58.0	72.0	15.1
	12	0.634	2.118	1.463	13.5	19.0	21.0	7.9	12	28.0	59.0	70.0	28.3
	13	0.069	2.388	1.119	9.5	18.0	25.0	2.7	13	25.0	50.0	68.0	35.5
	14	0.159	1.057	3.297	13.0	24.5	33.0	3.0	14	30.5	57.0	69.0	25.0
	15	0.082	0.312	1.376	16.5	22.0	26.0	5.0	15	26.5	49.0	61.0	19.3
	16	0.209	1.831	3.727	5.0	12.0	14.0	1.3	16	25.0	45.0	55.0	12.6
	17	0.099	1.166	3.682	18.0	29.5	32.0	9.6	17	28.0	65.0	77.0	21.4
	18	0.097	2.594	3.779	12.0	15.0	17.0	4.4	18	30.0	57.0	72.0	32.2
	19	0.313	1.875	2.459	22.0	22.0	26.0	5.5	19	22.0	40.0	53.0	9.8
	20	0.442	2.235	1.577	21.0	21.0	24.0	6.1	20	27.5	61.0	73.0	25.4
		0.283	**1.555**	**2.297**	**14.0**	**21.2**	**25.7**	**5.8**		**27.2**	**53.1**	**64.4**	**20.7**
		0.240	**0.635**	**1.243**	**2.8**	**4.0**	**5.1**	**2.1**		**2.0**	**5.3**	**7.0**	**7.0**

Line	N°	ELISA I	ELISA II	ELISA III	h1i	h2i	h3i	w3i	N°	h1c	h2c	h3c	w3c
93	1	0.181	0.708	2.674	18.5	35.5	43.0	13.8	1	26.0	54.0	65.0	23.2
	2	0.000	0.234	1.747	23.0	32.0	36.0	6.5	2	31.0	54.0	58.0	16.9
	3	0.177	1.090	1.057	22.0	33.5	37.0	4.0	3	34.0	67.0	80.0	19.3
	4	0.064	0.321	1.092	17.5	27.0	36.0	5.5	4	29.0	48.0	57.0	16.6
	5	0.004	0.560	1.629	20.0	32.0	36.0	6.9	5	28.5	58.0	67.0	30.9
	6	0.782	1.071	3.620	14.0	32.0	46.0	15.4	6	27.0	60.0	74.0	29.6
	7	0.281	0.307	1.474	22.0	33.0	38.0	8.8	7	29.0	50.0	59.0	22.5
	8	0.181	0.142	3.474	21.0	28.0	31.0	8.7	8	31.0	50.0	60.0	18.2
	9	0.535	0.225	2.349	19.5	26.5	41.0	4.4	9	35.0	58.0	71.0	13.5
	10	0.175	0.102	1.229	21.0	31.0	34.0	6.5	10	34.0	59.0	71.0	20.0
	11	0.071	0.497	ns	13.5	14.0	16.0	0.7	11	30.5	35.0	34.0	1.3
	12	0.046	0.093	3.432	15.0	30.5	36.0	7.8	12	29.0	52.0	66.0	17.2
	13	0.455	2.156	2.877	18.0	32.0	38.0	9.2	13	31.0	55.0	66.5	22.6
	14	0.008	0.099	1.013	22.0	39.0	41.0	9.0	14	33.0	53.0	62.0	12.5
	15	0.459	1.163	3.515	22.0	33.0	35.0	6.1	15	28.0	54.0	56.0	*5.9*
	16	0.278	0.245	3.496	18.0	26.0	27.0	2.8	16	27.0	41.0	52.0	7.8
	17	0.257	0.386	3.676	21.0	39.0	45.0	12.4	17	29.5	49.0	57.0	24.3
	18	0.501	0.314	2.255	11.0	21.0	24.0	3.7	18	31.0	55.0	64.0	21.9
	19	0.192	2.392	0.442	16.0	32.5	40.0	10.7	19	22.0	44.0	52.0	30.5
	20	0.295	0.587	1.131	19.0	38.0	44.0	14.8	20	23.0	40.0	51.0	15.7
		0.247	**0.635**	**2.220**	**18.7**	**30.8**	**36.2**	**7.9**		**29.4**	**51.8**	**61.1**	**18.5**
		0.162	**0.477**	**0.965**	**2.7**	**4.2**	**5.1**	**3.2**		**2.6**	**5.7**	**7.5**	**6.0**

5‘3‘AS First greenhouse resistance test

Line	N°	ELISA I	ELISA II	ELISA III	h1i	h2i	h3i	w3i	N°	h1c	h2c	h3c	w3c
125	1	0.030	0.467	2.078	17.0	20.0	24.0	6.1	1	22.0	48.0	59.0	27.6
	2	0.000	1.104	1.417	15.0	17.0	31.0	8.9	2	27.0	56.0	65.0	32.7
	3	0.634	0.354	1.019	13.5	24.5	30.0	6.3	3	14.5	34.0	41.0	9.7
	4	0.000	0.748	1.457	11.0	22.0	22.0	1.5	4	22.0	40.0	45.0	10.5
	5	0.324	2.355	1.256	18.0	24.0	41.0	8.3	5	19.0	38.0	50.0	15.5
	6	0.608	1.297	3.642	10.0	21.0	27.0	4.6	6	8.0	36.0	46.0	26.3
	7	0.045	1.043	1.269	11.5	23.5	31.0	4.6	7	20.0	41.0	56.0	16.6
	8	0.326	1.223	3.411	11.0	20.0	23.0	4.5	8	24.0	45.0	54.0	9.0
	9	0.407	1.507	3.654	12.0	22.0	24.0	2.0	9	10.5	29.0	40.0	10.4
	10	1.888	1.441	3.608	7.0	12.0	15.0	1.0	10	21.0	39.0	53.0	9.4
	11	1.061	1.236	3.904	9.5	17.0	18.0	5.0	11	19.0	39.0	54.0	17.3
	12	0.226	0.523	1.725	9.0	16.0	20.0	1.6	12	20.0	47.0	62.0	21.7
	13	0.125	1.352	3.481	8.0	17.0	22.0	7.4	13	26.0	54.0	70.0	32.2
	14	0.459	1.277	2.956	5.0	15.0	18.0	4.6	14	20.5	38.0	47.0	16.2
	15	0.864	1.474	3.593	6.0	12.0	15.0	1.8	15	4.0	10.0	25.0	6.2
	16	0.266	0.296	1.549	11.0	20.0	22.0	3.2	16	21.0	41.0	52.0	11.9
	17	0.121	1.272	3.569	8.0	15.0	19.0	3.1	17	22.0	44.0	54.0	17.2
	18	2.320	1.845	2.584	6.0	14.0	21.0	4.1	18	22.0	50.0	66.0	29.6
	19	0.458	1.427	1.987	5.0	9.0	12.0	0.7	19	11.0	16.5	30.0	3.8
	20	0.000	0.957	1.119	12.0	16.0	19.0	2.5	20	13.0	24.0	32.0	7.3
		0.508	**1.160**	**2.464**	**10.3**	**17.8**	**22.7**	**4.1**		**18.3**	**38.5**	**50.1**	**16.6**
		0.433	**0.379**	**0.976**	**2.9**	**3.6**	**4.9**	**1.9**		**4.9**	**8.2**	**9.4**	**7.2**

Infected controls for 5‘3‘S and 5‘3‘AS first greenhouse resistance test

Line	N°	ELISA I	ELISA II	ELISA III	h1i	h2i	h3i	w3i	N°	h1c	h2c	h3c	w3c
138	1	1.245	3.322	3.451	12.0	15.0	23.0	4.6	1	29.5	55.0	59.0	32.0
	2	0.051	2.065	1.026	11.5	14.0	21.0	5.1	2	28.0	47.0	63.0	14.6
	3	0.047	2.472	2.296	14.0	17.0	23.0	2.6	3	25.0	50.0	60.0	28.2
	4	0.851	3.125	2.789	13.5	18.0	21.0	3.6	4	23.5	40.0	42.0	6.8
	5	0.458	1.654	2.345	12.0	17.0	23.0	6.3	5	28.0	52.0	63.0	17.9
	6	0.032	1.325	1.344	15.0	22.0	27.0	7.1	6	25.0	53.0	69.0	21.4
	7	0.171	1.299	3.053	16.0	20.0	27.0	4.5	7	30.0	55.0	69.0	22.5
	8	0.745	1.865	3.139	6.0	13.0	16.0	0.8	8	18.5	36.0	46.0	8.1
	9	0.939	1.686	3.319	4.0	10.0	13.0	1.4	9	23.0	36.0	41.0	8.8
	10	0.940	2.820	2.655	12.0	21.0	24.0	3.1	10	24.0	42.0	56.0	10.7
	11	0.295	2.611	2.745	17.0	20.0	22.0	5.1	11	18.5	40.0	54.0	15.9
	12	0.579	2.569	3.412	15.0	17.0	21.0	4.7	12	19.0	37.0	46.0	8.8
	13	0.314	1.479	3.745	7.0	14.0	19.0	2.4	13	20.0	43.0	58.0	23.6
	14	0.451	1.021	1.747	12.0	25.0	27.0	5.0	14	20.0	46.0	63.0	17.8
	15	1.913	1.452	3.439	4.0	7.0	7.0	0.1	15	22.0	42.0	54.0	10.0
	16	0.021	1.023	1.328	10.0	16.0	22.0	1.7	16	19.0	33.0	42.0	11.5
	17	0.256	1.450	2.688	14.0	20.0	23.0	5.6	17	23.0	38.0	47.0	10.8
	18	0.062	2.448	3.683	15.0	21.0	26.0	6.0	18	30.5	58.0	70.0	31.9
	19	0.412	1.141	1.471	9.0	19.0	29.0	4.7	19	10.5	29.0	49.0	6.5
	20	0.299	1.704	3.448	6.0	16.0	24.0	5.1	20	14.0	46.0	64.0	31.0
		0.504	**1.927**	**2.656**	**11.2**	**17.1**	**21.9**	**4.0**		**22.6**	**43.9**	**55.8**	**16.9**
		0.368	**0.602**	**0.704**	**3.3**	**3.2**	**3.5**	**1.6**		**4.2**	**6.7**	**8.1**	**7.4**

Line	N°	ELISA I	ELISA II	ELISA III	h1i	h2i	h3i	w3i	N°	h1c	h2c	h3c	w3c
Nb	1	0.005	1.280	1.186	16.0	20.0	24.0	5.1	1	22.0	49.0	68.0	36.9
	2	0.266	1.245	1.334	12.0	19.0	23.0	2.3	2	21.0	47.0	61.0	17.5
	3	1.078	0.561	3.642	13.0	18.0	23.0	4.0	3	23.5	45.0	58.0	15.8
	4	1.303	2.504	3.689	9.0	19.0	24.0	1.6	4	27.0	54.0	69.0	19.9
	5	0.245	1.159	2.198	15.0	22.0	27.0	4.0	5	28.5	57.0	69.0	23.5
	6	0.510	1.229	1.882	17.0	19.0	21.0	4.6	6	32.0	63.0	77.0	25.7
	7	1.452	0.489	2.341	18.0	20.0	23.0	4.8	7	25.0	58.0	74.0	44.4
	8	0.705	0.715	3.438	8.0	19.0	23.0	1.4	8	28.0	57.5	70.0	19.4
	9	0.318	1.342	1.984	14.0	19.0	26.0	2.6	9	27.5	55.0	69.0	14.6
	10	0.238	2.662	1.479	13.0	22.0	23.0	1.9	10	22.0	40.0	52.0	8.3
	11	0.369	2.963	3.678	13.0	16.0	20.0	6.5	11	31.0	58.0	74.0	21.7
	12	0.334	1.880	3.819	4.0	9.0	10.0	0.6	12	20.5	43.0	61.0	16.4
	13	0.159	0.975	1.013	16.0	22.0	25.0	6.9	13	15.5	35.0	54.0	10.5
	14	0.460	1.899	1.030	13.0	18.0	23.0	3.1	14	30.0	57.0	71.0	20.0
	15	0.792	3.139	3.716	16.0	22.0	24.0	3.9	15	20.0	36.5	48.0	5.6
	16	1.173	1.433	3.562	13.0	24.0	26.0	2.5	16	17.5	32.0	47.0	6.3
	17	0.723	1.779	2.666	19.0	34.0	21.0	5.3	17	25.5	51.0	70.0	17.3
	18	0.259	0.720	2.635	12.0	17.0	23.0	4.1	18	21.0	39.0	57.0	11.5
	19	0.244	1.053	1.949	11.0	22.0	24.0	3.1	19	20.0	27.0	47.0	9.7
	20	0.260	1.457	2.135	4.0	8.0	15.0	1.2	20	11.0	24.0	36.0	3.7
		0.545	**1.524**	**2.469**	**12.8**	**19.4**	**22.4**	**3.5**		**23.4**	**46.4**	**61.6**	**17.4**
		0.341	**0.616**	**0.863**	**3.0**	**3.3**	**2.5**	**1.4**		**4.4**	**9.6**	**9.5**	**7.2**

ORF1/2 Second greenhouse resistance test

Line	N°	ELISA I	ELISA II	ELISA III	h1i	h2i	h3i	w3i	h1c	h2c	h3c	w3c
98	1	0.241	0.112	0.000	33	46	55	11.6	34	46	54	18.4
	2	1.869	1.564	1.671	26	28	32	3.9	37	48	56	12.9
	3	0.542	0.611	0.000	30	38	46	5.7	34	42	51	9.3
	4	2.722	3.672	3.680	20	25	31	4.5	16	24	26	2.1
	5	0.149	0.094	0.033	33.5	40	46	7.5	34	46	55	11.6
	6	0.231	0.541	0.142	32	40	45	4.8	34	49	61	11.3
	7	1.481	3.610	3.756	25	27	31	8.6	31	47	59	13.4
	8	1.059	3.700	3.366	23	29	34	3.8	33	45	53	12.8
	9	0.026	0.211	0.000	29	34	38	6.6	31	44	54	8.7
	10	0.141	0.214	0.000	35	41	48	8.3	34	45	53	6.9
	11	0.341	0.244	0.000	30	37	43	5.9	30	41	48	6.8
	12	0.068	1.111	0.296	30	36	42	8.1	33	46	56	17.3
	13	0.472	2.352	2.391	23.5	32	36	7.5	32	45	55	14.6
	14	0.784	0.141	0.000	28	36	43	6.9	36	50	60	10.8
	15	0.274	0.149	0.000	28.5	34	39	4.9	34	46	57	7.8
	16	0.545	0.274	0.000	30.5	37	46	9.4	38	51	61	9.7
	17	1.555	3.553	3.699	22	25	28	7.8	35	48	56	8.1
	18	0.926	2.780	2.877	24.5	31	39	6.9	35	50	59	20.4
	19	0.468	0.413	1.936	8	13	18	1.1	26	40	52	8.1
	20	0.220	0.353	0.889	8	12	19	1.6	25	38	49	9.1
		0.706	**1.285**	**1.237**	**26.0**	**32.0**	**38.0**	**6.3**	**32.1**	**44.6**	**53.7**	**11.0**
		0.546	**1.224**	**1.348**	**5.4**	**6.6**	**7.5**	**2.0**	**4.8**	**5.8**	**7.3**	**4.3**

Line	N°	ELISA I	ELISA II	ELISA III	h1i	h2i	h3i	w3i	h1c	h2c	h3c	w3c
108	1	0.475	0.142	0.128	33	45	54	17.4	35	48	57	11
	2	0.784	0.457	0.020	39	49	56	13.3	29	34	41	4.5
	3	0.198	0.793	0.780	28	35	43	10.4	30	40	45	5.9
	4	0.007	0.115	0.030	40	49	55	8.5	32	42	48	11.5
	5	0.054	0.214	0.000	36	44	51	8.7	27	37	42	4.7
	6	0.000	0.081	0.070	37	48	57	10.1	29	43	50	6.7
	7	1.268	3.682	3.000	29	36	43	10	34	47	43	9.9
	8	0.147	0.245	0.000	33.5	40	47	8	27	36	43	4.4
	9	0.245	2.038	3.303	22	28	36	7.5	28	37	43	5.5
	10	0.000	0.011	0.244	36	43	51	12.9	27	34	38	3.4
	11	0.116	0.569	0.675	29.5	36	44	9.1	28	38	45	5.5
	12	0.019	0.000	0.086	39.5	50	57	11	30	40	49	6.5
	13	0.109	0.548	0.381	21	29	38	16.3	29	44	55	14.6
	14	0.209	0.593	0.533	21	26	34	7.3	32	45	52	8.9
	15	2.398	2.296	2.330	22	27	32	5.6	33	46	56	10.3
	16	0.047	0.215	0.089	34.5	42	49	12.7	33	45	57	9.8
	17	3.320	3.458	3.600	18	20	25	4.3	34	46	56	9.5
	18	2.142	2.894	3.613	19	24	29	9.5	28	37	44	4.8
	19	0.746	1.241	2.246	11	22	30	6.6	27	37	46	9.7
	20	1.285	1.457	1.742	16	26	32	2.6	26	44	56	13
		0.678	**1.052**	**1.144**	**28.2**	**36.0**	**43.1**	**9.6**	**29.9**	**41.0**	**48.3**	**8.0**
		0.719	**0.970**	**1.183**	**7.6**	**8.7**	**8.9**	**2.8**	**2.7**	**4.4**	**6.0**	**3.1**

ORF1/2 Second greenhouse resistance test

Line	N°	ELISA I	ELISA II	ELISA III	h1i	h2i	h3i	w3i	h1c	h2c	h3c	w3c
110	1	0.000	0.112	0.231	24	27	33	7.4	30	43	50	12.6
	2	0.000	0.274	0.451	26	28	34	8.7	27	38	46	6.8
	3	0.211	0.358	0.745	20	25	28	4.7	29	38	44	6.6
	4	0.192	2.047	1.264	20	22	28	2.6	30	39	45	12.1
	5	0.000	0.451	0.612	14	20	22	7.9	25	40	51	15.7
	6	1.633	0.421	1.164	17	25	31	2.2	20	32	42	6.8
	7	1.108	0.651	1.045	12	17	20	2.3	27	36	39	6.5
	8	0.000	0.000	0.000	28	34	40	9.1	24	33	38	10.3
	9	0.000	0.000	0.000	27	30	37	9.7	23	32	40	5.9
	10	0.965	1.281	0.798	14.5	18	20	2.5	27	41	52	8.4
	11	1.493	2.186	2.778	19.5	24	28	4.6	30	42	50	10.1
	12	0.000	0.103	0.278	31	38	35	8.1	19	28	35	11.5
	13	1.231	3.383	3.519	16.5	21	28	5.7	28	41	50	16.4
	14	0.000	0.000	0.000	30	38	40	9.7	27	44	55	14.3
	15	0.744	2.329	1.749	23.5	28	32	6.6	33	48	59	23.5
	16	0.011	0.000	0.000	26.5	33	39	9.1	29	42	51	6.9
	17	1.101	1.970	2.103	12	15	20	1.7	27	40	48	8.3
	18	0.244	0.411	1.299	10.5	15	23	2.5	30	45	56	13.6
	19	0.613	1.702	1.621	10	15	17	1.3	14	23	39	8.6
	20	0.138	1.051	1.335	6	9	10	0.5	21	36	46	8.8
		0.484	**0.937**	**1.050**	**19.4**	**24.1**	**28.2**	**5.4**	**26.0**	**38.0**	**46.8**	**10.7**
		0.501	**0.846**	**0.739**	**6.2**	**6.5**	**6.7**	**2.8**	**4.5**	**5.9**	**6.4**	**4.3**

Line	N°	ELISA I	ELISA II	ELISA III	h1i	h2i	h3i	w3i	h1c	h2c	h3c	w3c
112	1	0.011	0.000	0.000	25	36	40	12	32	46	55	15.4
	2	0.149	1.713	0.947	24	30	35	7.8	29	42	51	16.4
	3	1.581	2.388	2.672	12.5	14	15	1.5	28	38	43	8.9
	4	0.000	0.000	0.000	17	24	31	10.3	27	34	43	7.8
	5	0.196	0.226	0.335	20	24	27	3.5	25	36	39	8
	6	0.011	0.471	0.345	18	20	24	5.8	26	33	37	10.2
	7	0.000	0.000	0.000	12	15	24	8.4	30	45	54	12.8
	8	0.000	0.000	0.000	27	32	37	9.4	27	35	39	7.9
	9	0.000	0.142	0.514	14	22	27	8.2	30	40	46	7.3
	10	0.013	0.005	0.000	29.5	37	42	10.1	24	32	36	9
	11	0.320	0.378	0.455	24	30	35	5.6	32	45	53	14.5
	12	0.399	1.877	3.121	14	16	20	9.1	28	42	51	17
	13	0.030	1.582	1.403	8	10	12	1	32	45	57	17
	14	0.000	0.548	0.874	13	20	23	13.7	24	34	39	7.2
	15	0.000	0.000	0.000	24	31	38	12	22	31	39	5.7
	16	0.000	0.714	0.678	23.5	30	36	9.7	24	34	42	7.9
	17	2.012	2.219	3.216	9	11	14	3.2	31	43	52	9.2
	18	0.338	2.045	2.478	6.5	9	14	1.7	26	34	44	14.6
	19	0.146	0.659	0.993	6	10	11	0.2	18	28	37	8.6
	20	0.187	0.813	1.919	8	13	18	1.4	21	32	39	7.2
		0.270	**0.789**	**0.998**	**16.8**	**21.7**	**26.2**	**6.7**	**26.8**	**37.4**	**44.8**	**10.6**
		0.330	**0.711**	**0.882**	**6.4**	**7.9**	**8.6**	**3.7**	**3.8**	**5.4**	**6.8**	**3.7**

ORF1/2 Second greenhouse resistance test

Line	N°	ELISA I	ELISA II	ELISA III	h1i	h2i	h3i	w3i	h1c	h2c	h3c	w3c
117	1	0.751	1.928	2.774	22	30	37	8.9	40	55	65	17.1
	2	1.142	0.917	0.973	26.5	33	36	10.2	36	46	53	8.1
	3	1.663	3.356	2.177	14	20	24	1	32	42	49	9.7
	4	0.783	1.746	0.919	25	30	34	8.7	35	46	54	11.5
	5	1.106	3.064	1.972	25	31	35	5.9	37	51	59	14.8
	6	0.864	3.604	2.011	27	34	42	11.6	32	52	64	21.7
	7	1.641	2.817	2.856	26	34	40	10.4	40	54	62	14.8
	8	2.565	1.986	1.900	20	26	32	4.1	32	44	53	7.9
	9	0.309	0.356	0.720	20.5	27	31	4.5	33	45	56	13
	10	0.951	2.670	2.038	24	30	34	6	29	39	48	7.2
	11	1.382	2.039	1.687	25.5	31	35	6.3	34	44	52	9.9
	12	2.616	1.891	1.910	22	30	35	5.4	29	41	49	7.1
	13	0.274	1.938	2.228	21	30	38	7.8	29	42	53	7.4
	14	0.236	1.915	2.318	24	29	34	9.2	27	39	47	5.7
	15	0.000	0.000	0.000	33.5	40	46	11.8	26	37	42	4.6
	16	0.000	0.000	0.051	32	38	44	10.5	27	37	42	4.3
	17	0.882	1.229	2.155	20	27	31	4.5	26	33	37	3.1
	18	0.529	0.882	1.079	20	28	36	9.9	20	27	37	2.8
	19	2.107	0.867	2.974	13	19	22	1.7	17	31	41	7.7
	20	2.270	0.683	0.702	18	27	32	3.3	23	40	54	11.9
		1.104	**1.694**	**1.672**	**23.0**	**29.7**	**34.9**	**7.1**	**30.2**	**42.2**	**50.9**	**9.5**
		0.656	**0.862**	**0.726**	**3.9**	**3.5**	**4.0**	**2.8**	**6.0**	**7.2**	**8.0**	**4.8**

Controls for ORF1/2 second greenhouse resistance test

Line	N°	ELISA I	ELISA II	ELISA III	h1i	h2i	h3i	w3i	h1c	h2c	h3c	w3c
158	1	1.916	1.875	3.200	22.5	23	27	5.3	28	45	54	15.6
	2	1.970	1.745	1.693	7	9	10	1.1	31	44	52	12.9
	3	0.987	1.354	1.987	23	26	30	6.6	38	50	58	16.1
	4	0.452	1.456	1.103	7.5	8	10	0.9	35	48	56	14.1
	5	0.387	1.745	1.659	21	27	27	4.4	34	49	57	15.9
	6	2.825	1.547	2.086	20	27	30	3.1	39	57	67	29.2
	7	0.121	1.958	2.851	15	22	22	3.3	26	43	54	8.6
	8	1.532	1.906	1.754	12	18	21	3.1	33	45	55	11.1
	9	1.826	2.296	2.687	15.5	21	24	3.3	12	20	27	2.2
	10	1.418	2.232	2.548	20	23	29	5.1	30	43	52	12.5
	11	0.727	1.587	1.784	14	21	24	4.5	37	52	62	14.9
	12	0.920	1.780	1.270	9	16	20	2.7	38	53	64	14
	13	0.714	2.654	2.946	10	18	21	3.5	21	33	43	9
	14	0.368	1.554	3.315	8.5	10	14	3.4	35	48	58	16.3
	15	1.359	2.293	2.670	15	19	20	2.1	21	32	43	9.1
	16	2.407	2.457	3.089	9.5	12	15	1.9	34	46	56	10.7
	17	0.555	1.810	1.826	17	22	26	4.7	37	50	60	11.8
	18	1.124	1.586	1.788	12	19	21	3.8	32	46	55	16
	19	0.202	1.554	1.885	16	26	30	4.1	18	33	43	9.8
	20	1.272	1.893	1.370	13	20	24	1.8	23	39	51	9.8
		1.154	**1.864**	**2.176**	**14.4**	**19.4**	**22.2**	**3.4**	**30.1**	**43.8**	**53.3**	**13.0**
		0.614	**0.279**	**0.590**	**4.1**	**4.5**	**4.8**	**1.1**	**7.4**	**8.5**	**8.7**	**5.0**

Line	N°	ELISA I	ELISA II	ELISA III	h1i	h2i	h3i	w3i	h1c	h2c	h3c	w3c
Nb	1	2.815	3.000	1.855	21	27	27	6.6	25	41	53	18.5
	2	1.235	1.118	0.956	22	24	25	2	32	48	55	11.4
	3	0.000	0.000	0.000	34	46	54	14.8	33	43	52	9.8
	4	1.442	3.406	3.387	18	23	28	3.1	33	45	52	10.2
	5	1.302	3.304	2.708	14	17	28	6.1	34	47	55	17.2
	6	1.653	3.112	0.957	18	24	28	5.2	33	50	63	16
	7	0.095	0.242	0.197	18	23	28	7.2	27	40	52	9.7
	8	1.457	1.857	2.145	14	19	24	4.7	33	46	56	12.9
	9	1.864	1.648	1.840	17	20	28	5	28	43	51	8.6
	10	0.392	1.274	3.297	9	15	20	4.3	34	47	55	8.2
	11	0.760	2.234	1.470	13	17	21	1.4	34	47	56	6.7
	12	0.539	3.111	2.299	15	20	27	4.7	30	46	59	13.3
	13	1.988	2.015	3.187	16	20	24	7.4	26	40	52	15
	14	0.787	0.560	2.755	14	21	27	7.1	29	44	54	10
	15	1.182	3.264	3.281	12	17	22	2.4	36	49	60	14.4
	16	1.132	3.250	2.441	12	16	20	1.4	32	47	55	8
	17	2.340	2.091	3.513	13	20	28	5.4	32	44	55	8
	18	0.236	3.332	2.767	16	23	25	4.7	27	40	51	8.7
	19	0.595	1.947	1.779	12	17	20	1.9	23	38	48	12.6
	20	0.351	2.092	3.161	14	21	29	4.5	22	38	47	10
		1.108	**2.143**	**2.200**	**16.1**	**21.5**	**26.7**	**5.0**	**30.1**	**44.2**	**54.0**	**11.5**
		0.622	**0.873**	**0.860**	**3.5**	**4.0**	**3.9**	**2.0**	**3.9**	**3.6**	**3.7**	**3.3**

5‘3‘S Second greenhouse resistance test

Line	N°	ELISA I	ELISA II	ELISA III	h1i	h2i	h3i	w3i	N°	h1c	h2c	h3c	w3c
31	1	1.939	1.925	3.432	15	23	32	8.3	1	31	46	56	20
	2	0.658	0.987	1.254	18	20	24	5.6	2	25	43	54	12.4
	3	0.005	0.000	0.000	28	46	58	16.7	3	33	51	60	20.4
	4	0.985	1.333	1.956	18	22	27	9.7	4	33	51	64	15.6
	5	2.113	1.864	2.818	10	20	28	4.3	5	26	45	56	10.8
	6	1.475	1.861	2.314	21	22	26	4.6	6	24	39	47	8.5
	7	0.785	1.044	1.335	16	22	30	5.8	7	23	39	50	6.3
	8	0.540	0.625	0.471	24	36	42	6.4	8	31	44	54	7.9
	9	0.954	1.054	1.845	14	20	22	5.3	9	28	41	49	7.3
	10	0.000	0.000	0.000	21	37	47	9.6	10	30	49	62	10.2
	11	1.120	1.358	1.743	13	30	37	7.2	11	30	46	59	14
	12	2.105	2.145	1.980	14	20	24	4	12	23	38	47	6.8
	13	1.728	1.905	2.104	15	19	25	6.1	13	30	46	58	9.5
	14	0.658	1.123	1.412	23	25	30	5.3	14	26	38	45	5
	15	0.014	0.000	0.000	24	39	48	11.8	15	28	47	62	12.1
	16	1.243	0.986	1.066	15	27	35	5.1	16	24	40	54	14.6
	17	0.000	0.000	0.000	25	39	46	14	17	27	43	56	12.9
	18	0.000	0.000	0.000	25	39	44	13.3	18	30	48	60	13
	19	1.235	1.845	2.355	16	22	27	4.6	19	30	47	61	15.3
	20	0.000	0.000	0.000	24	41	51	15.6	20	28	45	60	13.5
		0.878	**1.003**	**1.304**	**19.0**	**28.4**	**35.1**	**8.2**		**28.0**	**44.3**	**55.7**	**11.8**
		0.612	**0.643**	**0.895**	**4.4**	**7.9**	**9.2**	**3.4**		**3.1**	**4.0**	**5.5**	**4.1**

Line	N°	ELISA I	ELISA II	ELISA III	h1i	h2i	h3i	w3i	N°	h1c	h2c	h3c	w3c
33	1	0.000	0.000	0.000	30	48	54	14	1	34	51	62	17.7
	2	0.565	0.645	0.214	29	31	38	11	2	33	47	55	17.9
	3	0.000	0.000	0.000	30	35	47	9.2	3	35	50	58	17.4
	4	0.812	1.106	1.311	16	28	51	8	4	33	48	59	15.4
	5	2.201	1.507	0.645	22	37	39	8.2	5	30	49	60	16.3
	6	0.000	0.000	0.000	14	24	28	7.5	6	31	45	56	8.6
	7	2.327	1.959	0.582	22	33	39	8.9	7	33	48	60	10.9
	8	0.865	1.997	1.157	18	24	46	5.6	8	28	40	50	8.1
	9	2.123	1.790	1.247	20	31	33	6.4	9	35	49	58	9
	10	0.000	0.000	0.000	27	30	36	7.8	10	30	45	55	11.5
	11	1.661	0.712	0.632	20	28	39	8	11	32	46	56	10.5
	12	0.908	0.889	0.554	19	30	45	8.2	12	37	50	59	12.6
	13	1.321	0.967	0.539	23	33	45	8.2	13	33	52	62	12
	14	0.000	0.000	0.000	32	40	44	9.1	14	35	53	65	12
	15	0.145	0.338	0.356	28	39	43	9.6	15	32	49	60	13.5
	16	0.000	0.000	0.000	25	38	34	8.4	16	33	47	57	19.1
	17	1.637	1.639	1.253	24	28	35	8.1	17	33	49	60	13
	18	0.454	0.522	0.243	18	27	34	6.6	18	34	51	64	17
	19	2.586	2.724	1.089	17	27	59	15	19	30	45	56	12.9
	20	1.498	1.742	0.806	16	28	56	8.1	20	28	41	51	11
		0.834	**0.782**	**0.485**	**23.2**	**32.4**	**40.6**	**8.5**		**32.4**	**47.8**	**58.2**	**13.3**
		0.708	**0.622**	**0.395**	**4.4**	**4.8**	**5.6**	**1.2**		**2.3**	**3.3**	**3.7**	**3.2**

5‘3‘S Second greenhouse resistance test

Line	N°	ELISA I	ELISA II	ELISA III	h1i	h2i	h3i	w3i	N°	h1c	h2c	h3c	w3c
88	1	0.587	1.025	1.746	16	19	22	6.1	1	10	27	44	11.3
	2	1.145	1.784	1.956	19	25	28	5.4	2	17	36	46	12.7
	3	1.321	1.875	2.374	18	20	23	7.9	3	17	34	46	11.5
	4	0.000	0.000	0.000	15	27	40	8.3	4	14	27	39	12.9
	5	0.000	0.000	0.000	11	27	37	10.2	5	18	39	55	15.6
	6	1.662	1.989	2.354	10	15	15	2.8	6	14	31	44	7.8
	7	1.553	1.493	1.424	12	15	23	4.3	7	19	34	47	8
	8	0.986	1.125	1.345	9	14	20	3.8	8	16	28	38	5.4
	9	1.133	1.874	2.356	7	15	24	6.1	9	16	29	39	6.8
	10	0.000	0.030	0.000	17	29	41	9	10	12	29	43	7.5
	11	0.672	0.651	0.884	11	22	24	6.4	11	19	37	53	12.4
	12	0.962	1.547	1.457	13	17	20	5.1	12	21	35	45	8.5
	13	0.841	1.137	1.659	7	12	14	3.9	13	18	32	39	6.1
	14	1.421	1.775	2.745	8	11	12	3.4	14	14	29	39	5.9
	15	1.148	1.109	1.968	8	11	18	3.1	15	16	29	40	5.7
	16	2.468	2.474	2.539	5	17	21	1.8	16	17	35	51	13.5
	17	1.619	1.241	2.045	5	11	19	1.5	17	14	28	40	9.2
	18	1.654	1.587	1.873	12	17	20	3.2	18	21	40	52	15.7
	19	1.254	1.743	1.664	11	15	21	3.1	19	16	32	43	7.4
	20	0.652	1.125	1.425	8	19	26	6	20	13	30	45	9.6
		1.054	**1.279**	**1.591**	**11.1**	**17.9**	**23.4**	**5.1**		**16.1**	**32.0**	**44.4**	**9.7**
		0.479	**0.535**	**0.619**	**3.3**	**4.5**	**5.6**	**2.0**		**2.8**	**3.9**	**5.0**	**3.2**

Line	N°	ELISA I	ELISA II	ELISA III	h1i	h2i	h3i	w3i	N°	h1c	h2c	h3c	w3c
134	1	0.301	0.647	0.449	15	25	34	7	1	24	45	56	18.4
	2	0.005	0.174	0.333	23	32	45	10.1	2	27	46	58	20.9
	3	1.694	1.399	1.011	17	24	27	5.4	3	25	43	53	16.2
	4	1.421	1.363	0.442	17	30	48	11.7	4	26	45	57	19.5
	5	1.907	2.513	1.188	11	17	21	4.5	5	22	39	49	12.6
	6	1.073	0.942	0.998	17	28	38	6.8	6	25	44	58	10.5
	7	0.873	1.178	0.110	18	27	33	4.9	7	22	34	45	7
	8	1.548	1.005	0.109	24	32	40	11.9	8	27	38	46	6.6
	9	0.000	0.000	0.000	26	38	44	10.2	9	24	34	44	6.4
	10	1.928	0.971	1.224	15	19	23	6.2	10	22	39	51	10.1
	11	0.991	1.539	0.408	16	28	38	6.3	11	23	40	52	11.7
	12	0.068	0.129	0.235	25	34	41	9.7	12	27	42	50	9
	13	0.000	0.053	0.136	21	32	38	6.6	13	24	34	40	5.5
	14	0.590	0.461	0.252	12	16	38	8.1	14	25	42	55	10.6
	15	2.266	2.257	1.534	7	12	15	1.5	15	25	38	47	6.5
	16	0.671	1.126	1.549	14	19	24	11.8	16	19	37	48	10.9
	17	0.000	0.000	0.000	19	33	40	12.1	17	24	42	54	11.7
	18	0.000	0.000	0.000	24	37	44	13.9	18	23	43	56	17.9
	19	1.641	3.245	2.144	14	19	22	4.7	19	28	46	58	14.6
	20	0.000	0.048	0.321	22	37	44	9.3	20	21	39	50	13
		0.849	**0.953**	**0.622**	**17.80**	**27.0**	**34.8**	**8.1**		**24.2**	**40.5**	**51.4**	**12.0**
		0.685	**0.707**	**0.529**	**4.1**	**6.5**	**8.00**	**2.7**		**2.2**	**3.8**	**5.1**	**4.5**

5‘3‘ Second greenhouse resistance test

Line	N°	ELISA I	ELISA II	ELISA III	h1i	h2i	h3i	w3i	N°	h1c	h2c	h3c	w3c
135	1	1.793	2.165	1.994	8	15	20	3	1	25	46	59	22.5
	2	0.906	1.352	2.783	11	24	34	6.2	2	28	45	57	11.3
	3	0.711	0.658	0.766	16	26	33	7.1	3	21	35	42	12.1
	4	1.081	1.461	1.239	10	16	24	3.1	4	26	45	56	22.5
	5	0.517	0.698	0.702	10	19	30	7.3	5	24	38	46	13
	6	0.646	0.764	0.622	6	10	17	1.9	6	22	41	56	11
	7	0.712	2.061	3.188	11	16	20	2.7	7	27	44	55	11.1
	8	0.293	1.199	0.841	18	28	33	6.1	8	24	38	46	8.9
	9	1.025	1.471	1.276	14	23	28	2.7	9	23	39	49	8.4
	10	1.266	1.353	2.229	15	23	30	6.6	10	24	43	52	11.4
	11	1.102	1.657	1.785	12	21	32	8.1	11	23	44	59	15.4
	12	1.058	1.168	0.897	12	20	28	3.6	12	27	41	51	12.4
	13	1.177	1.688	1.943	13	19	25	2.5	13	18	36	46	6.4
	14	0.986	1.272	1.250	12	15	19	2.2	14	22	40	53	9.6
	15	1.147	1.787	1.517	14	21	28	2.6	15	25	41	43	4.7
	16	1.708	1.549	2.620	12	22	32	4.4	16	21	43	57	17
	17	1.311	1.372	0.960	11	20	27	4.8	17	20	40	50	14.7
	18	1.317	1.253	2.813	9	16	22	2.7	18	17	35	45	11.5
	19	1.100	0.803	0.994	14	25	35	7.1	19	22	39	48	8.6
	20	0.978	1.235	1.123	24	27	32	6.8	20	24	40	51	14.9
		1.042	**1.348**	**1.577**	**12.6**	**20.3**	**27.4**	**4.6**		**23.1**	**40.6**	**51.0**	**12.4**
		0.260	**0.308**	**0.674**	**2.7**	**3.7**	**4.6**	**1.9**		**2.8**	**3.2**	**5.2**	**4.5**

5‘3‘AS Second greenhouse resistance test

Line	N°	ELISA I	ELISA II	ELISA III	h1i	h2i	h3i	w3i	N°	h1c	h2c	h3c	w3c
28	1	0.000	0.000	0.000	23	41	53	15.5	1	24	41	50	12.4
	2	0.000	0.000	0.000	26	43	56	12.8	2	21	35	41	11.9
	3	0.954	1.548	2.145	17	19	22	8.2	3	29	42	52	16.4
	4	0.000	0.000	0.000	24	40	50	10.7	4	32	46	54	16.3
	5	1.234	1.985	2.355	14	20	23	5.4	5	28	46	58	15.1
	6	0.000	0.000	0.000	19	34	45	6.7	6	29	47	55	10.6
	7	0.546	1.358	2.145	14	20	25	5.5	7	29	46	59	13.9
	8	0.987	1.365	1.987	12	18	24	4.1	8	24	36	45	6.4
	9	0.230	1.111	1.745	15	19	21	4.6	9	32	47	58	14.3
	10	0.988	1.487	1.994	14	15	19	3.3	10	34	48	58	12.7
	11	1.235	1.654	2.335	9	14	14	3.1	11	27	44	55	12.5
	12	0.988	1.985	2.874	10	12	16	5.3	12	19	33	43	8
	13	0.000	0.000	0.000	24	36	43	6.9	13	32	46	55	14.3
	14	0.548	0.945	0.879	20	31	39	4.7	14	29	43	53	9.7
	15	0.894	1.363	2.014	15	17	20	2.1	15	31	43	53	13.8
	16	0.000	0.000	0.000	30	41	51	16.4	16	29	41	50	9.6
	17	0.986	1.665	2.621	16	18	22	2.7	17	31	46	55	18.2
	18	0.460	0.569	0.336	25	36	42	7.7	18	33	47	57	15.4
	19	0.352	0.641	0.455	22	34	42	6.7	19	26	37	47	8.5
	20	0.978	1.654	1.784	17	20	24	2.3	20	24	37	45	10.9
		0.569	**0.967**	**1.283**	**18.3**	**26.4**	**32.6**	**6.7**		**28.2**	**42.6**	**52.2**	**12.6**
		0.412	**0.654**	**0.988**	**4.8**	**9.8**	**12.8**	**3.1**		**4.0**	**4.5**	**5.3**	**3.0**

Line	N°	ELISA I	ELISA II	ELISA III	h1i	h2i	h3i	w3i	N°	h1c	h2c	h3c	w3c
77	1	0.000	0.000	0.000	25	40	51	12.3	1	21	38	46	12.6
	2	0.000	0.000	0.000	27	41	51	12.5	2	23	41	49	11.8
	3	0.785	0.687	0.693	14	22	31	4.3	3	27	43	49	11.1
	4	0.530	1.025	0.736	16	24	27	6.3	4	27	45	54	14.2
	5	0.007	0.687	1.004	14	20	23	5.7	5	20	41	54	11.4
	6	0.013	0.452	0.784	17	20	27	5.3	6	22	40	50	9.9
	7	0.912	0.476	0.145	13	18	22	3.9	7	24	38	46	9.6
	8	0.535	1.188	0.138	20	24	30	6.6	8	22	35	41	8.7
	9	1.528	1.097	0.542	15	21	27	5.9	9	23	37	47	7
	10	0.122	0.542	0.668	21	24	31	5.8	10	26	45	57	12
	11	0.000	0.000	0.000	23	34	40	6.9	11	21	34	42	6.2
	12	0.041	0.393	0.112	12	15	15	3.9	12	23	36	42	8.4
	13	0.040	0.192	0.784	8	14	14	3.7	13	28	42	49	7.5
	14	1.300	1.665	1.780	11	16	19	1.7	14	24	38	45	8
	15	0.084	0.644	0.978	14	20	21	3.7	15	23	36	46	8.1
	16	0.015	0.478	1.123	16	19	25	4.3	16	23	38	48	10.2
	17	0.741	0.871	0.897	19	24	27	4.8	17	23	41	52	11.6
	18	0.548	1.023	1.045	14	21	25	5.1	18	24	40	50	13.6
	19	0.000	0.000	0.000	21	37	43	7.5	19	24	41	51	9.4
	20	0.661	0.947	0.927	18	20	25	4.6	20	25	42	52	13.4
		0.393	**0.618**	**0.618**	**16.9**	**23.7**	**28.7**	**5.7**		**23.6**	**39.6**	**48.5**	**10.2**
		0.400	**0.365**	**0.401**	**3.9**	**5.8**	**7.6**	**1.8**		**2.0**	**3.04**	**4.1**	**2.3**

5‘3‘AS Second greenhouse resistance test

Line	N°	ELISA I	ELISA II	ELISA III	h1i	h2i	h3i	w3i	N°	h1c	h2c	h3c	w3c
86	1	0.541	0.987	1.144	14	22	27	6.6	1	31	47	57	16.3
	2	0.985	1.123	1.420	20	27	33	8.5	2	29	45	56	10.8
	3	0.475	1.417	1.745	14	20	26	6.7	3	32	48	60	16.7
	4	0.000	0.000	0.000	28	42	53	12.7	4	31	47	56	17.3
	5	0.074	1.578	2.145	17	19	22	3.2	5	33	51	61	19
	6	0.984	1.132	1.984	14	21	24	5.3	6	31	49	61	13
	7	0.597	0.717	3.606	15	21	25	4.8	7	34	50	60	13.7
	8	3.209	3.236	3.595	11	15	17	2.4	8	29	41	45	7.8
	9	1.739	3.303	0.784	19	23	34	4.4	9	28	40	52	9.4
	10	1.124	1.874	1.785	13	20	27	3.6	10	28	44	54	9.9
	11	0.546	0.984	0.784	21	26	34	4.3	11	26	40	51	8.3
	12	0.478	1.023	1.125	14	20	23	4.1	12	27	39	48	8.2
	13	1.036	1.250	1.831	9	12	16	1.7	13	29	44	55	8.7
	14	0.829	1.361	1.540	14	25	25	6.7	14	11	16	22	1.8
	15	1.401	3.382	0.808	11	19	22	2.8	15	32	48	60	13.7
	16	0.543	0.687	0.874	24	26	31	3.8	16	27	45	57	11.3
	17	0.787	1.065	1.144	22	27	34	6.2	17	26	44	55	13
	18	1.378	2.462	3.041	15	22	30	5.8	18	25	40	52	14
	19	0.678	0.741	0.541	19	24	33	7.6	19	19	33	40	8.4
	20	1.010	1.584	2.841	12	18	20	3.6	20	29	43	55	14.8
		0.921	**1.495**	**1.637**	**16.3**	**22.4**	**27.8**	**5.2**		**27.8**	**42.7**	**52.8**	**11.8**
		0.458	**0.695**	**0.784**	**4.0**	**4.0**	**5.9**	**1.9**		**5.1**	**7.5**	**8.8**	**4.0**

Line	N°	ELISA I	ELISA II	ELISA III	h1i	h2i	h3i	w3i	N°	h1c	h2c	h3c	w3c
93	1	0.011	0.987	1.897	19	27	31	8.1	1	28	43	53	18.2
	2	0.000	0.000	0.000	31	42	49	1.29	2	27	44	52	15.8
	3	0.674	0.557	0.775	21	34	44	10	3	25	41	52	12.7
	4	0.738	0.618	0.463	20	33	42	10	4	24	40	52	11.7
	5	1.019	0.636	2.234	17	28	38	8.8	5	20	32	40	14.8
	6	1.606	0.157	0.951	19	29	37	8.1	6	29	43	53	13.3
	7	0.024	0.227	0.214	26	39	47	10	7	18	30	39	5.6
	8	0.008	1.141	1.458	24	27	31	8.1	8	25	40	49	10.9
	9	2.417	3.636	3.483	18	24	31	5	9	25	40	50	10.3
	10	1.997	2.694	3.771	12	19	24	4.2	10	29	43	53	12.9
	11	1.419	1.271	1.589	16	22	27	3.2	11	27	45	55	10.5
	12	1.458	2.977	3.287	13	18	25	3.4	12	24	36	48	7.9
	13	0.987	1.354	1.987	16	20	23	9.2	13	30	42	51	7.9
	14	0.874	1.124	1.656	14	24	35	7.8	14	26	42	53	11.8
	15	0.869	0.845	0.594	23	28	34	8.4	15	24	36	45	7.7
	16	0.634	0.598	1.662	17	28	36	9.1	16	27	44	57	15.5
	17	1.123	1.054	1.155	21	24	33	8.5	17	28	45	55	10.7
	18	2.558	2.844	3.535	12	18	21	4.3	18	28	44	56	14.7
	19	3.262	3.438	3.289	18	28	35	8.9	19	17	38	48	9.3
	20	1.299	1.792	1.590	16	26	33	6.7	20	23	39	49	17.8
		1.149	**1.398**	**1.780**	**18.6**	**26.9**	**33.8**	**7.2**		**25.2**	**40.4**	**50.5**	**12.0**
		0.683	**0.900**	**0.925**	**3.6**	**4.7**	**5.9**	**2.2**		**3.5**	**4.1**	**4.7**	**3.3**

5‘3‘AS Second greenhouse resistance test

Line	N°	ELISA I	ELISA II	ELISA III	h1i	h2i	h3i	w3i	N°	h1c	h2c	h3c	w3c
125	1	1.311	2.292	2.005	7	14	20	3.3	1	15	31	46	15.1
	2	0.572	0.923	0.968	7	16	26	4.5	2	12	39	51	16.1
	3	2.482	1.466	3.445	14	23	32	8.5	3	19	28	41	10.6
	4	1.300	2.203	3.155	6	13	18	3	4	13	45	55	20.5
	5	0.987	1.256	1.578	10	23	23	7.4	5	26	30	43	14.5
	6	0.025	0.774	0.954	12	25	27	8.1	6	10	30	40	6.2
	7	2.810	2.074	3.397	14	26	32	6.7	7	15	26	32	6.8
	8	1.123	1.589	1.745	16	23	26	6	8	15	19	27	4.9
	9	1.312	2.251	2.524	7	15	22	5.8	9	14	33	40	5.5
	10	0.564	2.902	0.717	4	11	19	2.9	10	22	22	31	2.8
	11	0.090	0.875	1.457	14	18	21	4.3	11	11	35	48	13.2
	12	1.020	0.987	2.038	3	6	13	1.4	12	18	28	35	4.3
	13	1.610	1.988	2.457	6	11	16	3.4	13	16	20	30	4.8
	14	0.856	2.500	2.646	4	8	11	0.8	14	18	28	35	4.2
	15	2.496	3.302	3.684	2	5	7	0.9	15	13	23	29	4
	16	0.371	0.646	0.603	8	20	25	4.3	16	9	23	33	6
	17	0.783	2.622	2.500	8	15	20	3.2	17	12	29	40	5.5
	18	1.435	2.484	0.618	11	23	30	6.3	18	19	36	50	14.8
	19	0.412	0.578	0.568	16	29	36	5.6	19	11	23	34	5.4
	20	3.423	1.954	3.900	10	17	24	6.5	20	25	40	51	15.2
		1.249	**1.783**	**2.048**	**9.0**	**17.0**	**22.4**	**4.6**		**15.6**	**29.4**	**39.5**	**9.0**
		0.694	**0.696**	**0.928**	**3.6**	**5.7**	**5.7**	**1.9**		**4.6**	**6.8**	**8.2**	**5.2**

Controls for 5‘3‘S and 5‘3‘AS Second greenhouse resistance test

Line	N°	ELISA I	ELISA II	ELISA III	h1i	h2i	h3i	w3i	N°	h1c	h2c	h3c	w3c
138	1	0.828	1.044	2.057	13	20	22	6.2	1	20	34	43	13
	2	0.541	1.021	1.845	15	20	24	5.3	2	23	40	51	14.7
	3	1.226	0.132	1.721	12	19	27	4.1	3	25	43	54	17.3
	4	0.451	1.023	2.135	14	19	23	5.4	4	18	32	43	10.3
	5	2.140	1.198	3.214	14	17	22	3.9	5	20	43	57	13.6
	6	1.993	1.379	0.921	9	15	21	2.2	6	23	38	49	7.4
	7	2.364	1.255	1.198	6	12	14	2	7	20	36	48	7.4
	8	0.863	1.127	1.822	10	16	21	2.2	8	13	23	33	3.4
	9	1.821	2.873	1.122	4	8	11	1.2	9	23	36	46	6.9
	10	1.942	1.824	1.179	8	16	21	2.7	10	25	42	52	17.5
	11	0.879	3.176	1.763	10	18	23	5.1	11	24	43	53	12.6
	12	1.663	1.124	1.478	13	19	20	2.1	12	26	42	51	11.4
	13	0.978	1.689	2.356	15	19	21	4.1	13	17	25	34	5.1
	14	1.656	1.568	0.641	14	22	28	4.5	14	18	32	42	5.1
	15	0.730	2.169	2.181	9	22	29	4	15	15	33	48	11.1
	16	1.366	1.325	2.145	14	22	29	6.2	16	27	45	57	26.6
	17	1.545	0.801	1.713	15	23	28	7.1	17	17	37	49	10.1
	18	1.256	2.133	2.845	16	20	20	7	18	15	23	33	8
	19	0.540	2.379	0.698	12	23	30	6	19	20	42	54	14.4
	20	1.038	0.420	1.452	15	20	27	6.1	20	9	22	34	4.6
		1.291	**1.483**	**1.724**	**11.9**	**18.5**	**23.0**	**4.4**		**19.9**	**35.6**	**46.6**	**11.0**
		0.487	**0.595**	**0.512**	**2.7**	**2.8**	**3.8**	**1.5**		**4.6**	**7.2**	**7.7**	**5.4**

Line	N°	ELISA I	ELISA II	ELISA III	h1i	h2i	h3i	w3i	N°	h1c	h2c	h3c	w3c
Nb	1	2.455	1.950	2.251	12	22	24	5.2	1	23	43	55	20.1
	2	1.541	1.244	1.296	11	20	24	5.2	2	25	41	52	14.4
	3	1.668	1.798	1.718	13	20	21	5.2	3	29	46	58	22.2
	4	1.629	2.840	2.979	13	21	21	4.2	4	24	43	57	17.5
	5	1.766	2.584	2.155	10	19	26	3	5	20	40	54	16.5
	6	0.909	1.634	1.591	14	21	21	5.8	6	25	39	48	8.8
	7	1.279	1.274	1.606	12	19	25	2.2	7	24	37	49	8.2
	8	0.853	2.211	2.458	16	24	24	4.4	8	22	33	42	7.4
	9	1.624	1.958	2.406	12	20	25	3.3	9	26	44	55	12
	10	0.759	0.854	0.696	13	23	27	5.9	10	19	33	50	9.1
	11	0.400	0.909	1.872	8	13	20	1.7	11	23	40	53	10.6
	12	0.966	1.875	2.524	14	24	24	4.8	12	26	41	50	8.5
	13	1.422	1.511	2.546	14	18	20	3.6	13	24	39	49	7.4
	14	1.411	1.580	1.304	13	23	24	3.1	14	25	45	57	12
	15	1.835	2.144	2.494	11	21	27	3.1	15	25	42	51	8.5
	16	1.360	1.124	1.292	12	20	26	2.3	16	25	45	60	17.8
	17	1.437	1.308	3.339	10	18	24	2.4	17	20	38	50	11
	18	1.280	1.399	0.944	9	19	27	3.5	18	18	35	45	10
	19	0.856	2.318	2.237	12	21	28	4.5	19	18	36	48	10.8
	20	0.987	1.658	2.136	14	20	23	17	20	23	43	55	12
		1.322	**1.709**	**1.992**	**12.2**	**20.3**	**24.0**	**4.5**		**23.2**	**40.2**	**51.9**	**12.2**
		0.361	**0.430**	**0.561**	**1.5**	**1.7**	**1.9**	**1.7**		**2.8**	**3.8**	**4.4**	**4.3**

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